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MANUAL OF CLINICAL LABORATORY METHODS

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By

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TO
JAMES PERSONS SIMONDS

Preface

This book is the outgrowth of an outline of laboratory methods prepared for the use in the teaching of medical students and laboratory technicians. It is not designed to be a textbook of Clinical Pathology in that it does not discuss, except incidentally, the clinical significance of the results of the tests. In most instances only one method is included for each determination. The procedures are given in outline form so that they may be easily followed step by step in the laboratory.

The author especially desires to express her appreciation and thanks to Dr. J. P. Simonds for his encouragement and helpful suggestions in the preparation of the manuscript; to Dean J. R. Miller for his interest in the book; and to all the individuals whose interest and suggestions have been of assistance. Special thanks are given to Miss Edna Murmann who carefully proofread the manuscript and made many helpful criticisms, and to Miss Lucille Cassell and Miss Rosamond Howland who made most of the illustrations. The author is deeply grateful to the following technicians for valuable suggestions: Mrs. Arlene LeSuer, Miss Dorothy Siemsen, Miss Annie Laurie Peeler, and Miss Helen Gurley on Chemistry; Mrs. Eleanor Maynard on Hematology; Miss Eleanor Strack on Electrocardiography; Miss Alyce Anderson and Miss Esther Cheatle on Tissue Sectioning; and to Miss Betty Matz and Mrs. Jean Zieke for help in typing the manuscript.

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Opal E. Hepler

Foreword

The clinical laboratory is an essential part of current medical practice. Through its use, modern medicine is approaching the status of an exact science. The extent and manner of use of the clinical laboratory is a measure of the quality of medical service rendered by the staff of any hospital. Since the actual work of such laboratories is usually done by technicians with varying degrees of training and experience, the more detailed and explicit the available instructions for the performance of the various tests, the more accurate and dependable the results of such tests are likely to be. This *Manual of Laboratory Procedures* is intended to be just such a guide.

As stated in the Preface, this Manual is the outgrowth of an outline prepared several years ago for use in the laboratory by medical students in the course in Clinical Pathology in Northwestern University Medical School. By gradual accretion it has grown to its present size. The method of presentation of the subject matter is the result of the author's extensive experience in teaching medical students and student technicians and in directing the clinical laboratories of the Montgomery Ward Clinics and of Passavant Memorial Hospital. This method of presentation is based upon the principle that explicit directions are necessary, particularly for beginners, if laboratory tests are to be done with expedition and accuracy. Each test is, therefore, treated as a unit; that is, the principle of the test, the various steps in the order of their performance, the calculation of results and their interpretation and significance, the sources of error and the formulae of the solutions used, are all given in systematic order and in simple language. This step by step method is a unique feature of this Manual.

This Manual has gone through several previous editions in planograph form. Of the last edition there were four reprintings. Without advertising of any kind and without review in any medical journal, the demand for it became so great that its publication in book form seemed desirable. It has been used in many civilian hospitals throughout the United States. During World War II requests for it came from numerous military hospitals in South America, North Africa, Italy, France and the Western and Southwest Pacific areas. It has, therefore, proved its value under the most varied working conditions. The present edition is much larger and more extensively illustrated than any previous one and its usefulness should be proportionately enhanced. The illustrations and diagrams of different types of complicated apparatus—such as the electrocardiograph and the Van Slyke machine—should enable any technician to understand the principle upon which the particular apparatus is based and to use it with increasing assurance and accuracy.

This is not a textbook of Clinical Pathology. Its sole purpose is to improve the work of laboratory technicians and medical students by furnishing them with explicit, step-by-step directions for the performance of the different tests. References to the practical application and clinical significance of a particular test are inserted for the purpose of stimulating the interest of the technician in her work. Intelligent employees do better work if they understand the use that will be made of the product of their efforts.

James P. Simonds

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MANUAL OF CLINICAL LABORATORY METHODS

Urinalysis

TABLE 1. RECORDING ROUTINE URINALYSIS

COLOR: Colorless, pale straw, straw, light amber, amber, reddish amber.
SP. GR.: 1.001-1.060.
REACTION: pH determined with nitrazine paper.
APPEARANCE: Clear, white or orange flocculent sediment, or shreds.
CLOUDINESS: Amount present; + (slight), + + (moderate), + + + (cloudy), + + + + (very cloudy).

ALBUMIN: Ring test.
Neg. = no cloudiness appears at zone of contact.
Tr. = ring is just perceptible against a black background (less than 0.05 gm.%).
+ = ring is distinct against a black background and can barely be seen when held up to the light (0.05 to 0.2 gm.%).
+ + = ring is very definite against light, faintly visible when viewed from above (0.2 to 0.5 gm.%).
+ + + = ring is heavy against light, distinct cloudiness when viewed from above (0.5 to 2 gm.%).
+ + + + = ring is thick and dense against light, opaque when viewed from above (2 gm.% and over).

Heat test.
Neg. = no cloudiness is perceptible.
Tr. = cloudiness is just perceptible against a black background (less than 0.01 gm.%).
+ = cloudiness is distinct but not granular against a black background and can barely be seen when held up to the light (0.01 to 0.05 gm.%).
+ + = cloud is distinct and granular against light (0.05 to 0.2 gm.%).
+ + + = cloud is heavy with distinct flocculi (0.2 to 0.5 gm.%).
+ + + + = cloud is dense with large flocculi, may solidify (0.5 gm.% or higher, 3 gm.% albumin becomes solid on boiling).

SUGAR: Benedict's test (read before precipitate settles).
Neg. = clear blue to marked cloudy green.
+ = yellowish green (0.5 to 1 gm.%).
+ + = greenish yellow (1 to 1.5 gm.%).
+ + + = yellow (1.5 to 2.5 gm.%).
+ + + + = orange (2.5 to 4 gm.%), red (4 gm.% and over).

ACETONE: Specimens positive for sugar and all specimens from prenatal patients and patients in acidosis must be examined routinely for acetone.

DIACETIC ACID: Test only specimens positive for acetone.

MICROSCOPIC EXAMINATION (sediment of centrifuged specimen):
CASTS: Identify type—hyaline, finely granular, coarsely granular, etc. Report number on slide or average per low power field.
ERYTHROCYTES and LEUKOCYTES: Average range per high power field.
EPITHELIUM (low power): + (occasional), + + (few), + + + (many), + + + + (great many). Identify type—squamous, transitional, renal, etc.
CRYSTALS (low power): + (occasional), + + (few), + + + (many), + + + + (great many). Identify type—calcium oxalate, uric acid, etc.
AMORPHOUS (low power): + (occasional), + + (few), + + + (many), + + + + (great many). Urates in acid, phosphates in neutral or alkaline urine.
BACTERIA (high power) and MUCOUS THREADS (low power): + (occasional), + + (few), + + + (many), + + + + (great many).

General Considerations

The primary function of the kidney is to maintain the chemical and physical qualities of the blood plasma within normal limits by excreting metabolic wastes, especially those of nitrogenous composition. The fluid filtered from the blood plasma through the glomeruli contains all the substances present in the blood except protein and the cellular elements. As this filtrate passes down the tubule, the water is almost completely reabsorbed and the solid elements in solution (glucose, bicarbonate, phosphates, urea, sodium, and potassium) are selectively reabsorbed in varying amounts according to each individual threshold value. In this manner about 150 liters of plasma are purified each day by the process of glomerular filtration and tubular absorption. It is estimated that each kidney has a million nephrons (glomeruli with tubules) only a part of which act at one time; therefore in normal kidneys there is a reserve of 60 to 75 per cent.

Collection of Urine Specimen

I. Single Specimen.

A. *Qualitative Tests.*

1. When it is desired to make only qualitative tests, a specimen voided at random is satisfactory. It should be examined within 1 to 2 hours after voiding.
2. Urine passed about 3 hours after a meal is most likely to contain pathological substances.
3. First urine voided in the morning is least likely to contain them.
4. To diagnose cyclic albuminuria, samples obtained at various intervals during the 24 hours must be examined.

B. *Bacteriological Examinations.*

1. The urine must be a fresh, catheterized specimen obtained under aseptic conditions.
2. If cultures cannot be made immediately, place specimen in the refrigerator.

II. Twenty-four Hour Specimen.

A. *Quantitative tests* are of value only on 24 hour samples.

B. *Collection.*

1. The patient should be instructed to empty the bladder at the beginning of the period (8 A.M.) and discard the urine.
2. Save all urine passed until 8 o'clock the next morning, emptying the bladder at that time and adding this urine to the 24 hour specimen.
3. It is sometimes desirable to have the day and night specimens examined separately.
   a. *Day specimen*—obtain as for the 24 hour specimen, including the specimen voided 3 hours after the evening meal.
   b. *Night specimen*—save all urine voided during the night and empty bladder at 8 A.M., adding this to the night specimen.
4. If the amount is important, the patient should bring the pooled specimens to the laboratory; if not, 8 ounces of the specimens, well mixed, are sufficient.
5. The urine should be kept in a clean receptacle and in a cool place.

Physical Properties

I. Quantity.

A. *Normal.*

1. The 24 hour specimen for adults contains 800 to 1600 cc. (varies greatly with the liquid intake, perspiration, etc.).
2. Specimens from children, 6 to 12 years, contain 500 to 1500 cc.
3. Specimens from children, 1 to 6 years, contain 300 to 1000 cc.
4. The day volume is usually 3 to 4 times the night volume.

B. *Abnormal.*

1. *Increased quantity (polyuria)* is found in diabetes mellitus and insipidus, chronic nephritis, certain nervous diseases, during disappearance of an edema, and during convalescence from an acute febrile disease.
2. *Decreased quantity (oliguria)* is found in uremia, acute nephritis, eclampsia, severe diarrhea, excessive vomiting, profuse sweating in fevers, cardiac decompensation, calculus or tumor of the kidney, nephrosis with edema, atrophic hepatic cirrhosis, and acute yellow atrophy of the liver.
3. *Total suppression (anuria)* occurs in "collapse" with systolic blood pressure below 70 mm. of mercury, severe acute nephritis, and in poisoning with bichloride of mercury.
4. *Residual urine* is that obtained by catheter immediately after the patient has emptied the bladder voluntarily.

II. Color.

A. *Normal.*

1. *Straw to amber.*
2. *Colorless to straw* indicates low specific gravity and large quantity (except in diabetes mellitus).
3. *Amber* indicates high specific gravity and small quantity.

B. *Pathological Coloration.*

1. *Reddish amber* may indicate an increase in urobilinogen or porphyrin.
2. *Brownish yellow or green* with a yellow foam when shaken may indicate bile pigments.
3. *Red to smoky brown* may be due to blood and blood pigments.
4. *Milky* may be due to large amounts of pus, bacteria, fat, or chyle.
5. *Brownish black* may indicate melanin which may appear only after the urine stands and the chromogen melanogen is converted into melanin. If present a gray precipitate, which blackens on standing, forms when a few drops of 10% ferric chloride is added to 10 cc. of urine.
6. *Black* may indicate homogentisic acid which occurs in alkaptonuria. Urine becomes black after standing or after it is alkalinized. Homo-

gentisic acid reduces Benedict's solution.

C. ***Nonpathological Coloration***—following the ingestion of various drugs and foods.

1. *Red*—beets, mercurochrome or prontosil instillation, phenolphthalein, selenium.
2. *Blue or green*—methylene blue.
3. *Brown*—rhubarb, senna, cascara, argyrol instillation.
4. *Yellow*—carotene, santonin, or pyridium.
5. *Green*—acriflavine.

III. Specific Gravity.

A. ***Method.***

1. Fill the container three-fourths full of urine, remove all foam with filter paper.
2. Float the urinometer in the urine by rotating it rapidly to prevent its touching the bottom or the sides of the container.
3. The specific gravity is obtained by reading the gradation on the stem of the instrument at the level of the lower part of the meniscus.
4. Each urinometer is calibrated to give readings at a definite temperature, usually 25°C. If the temperature of the urine is above or below this, a correction of 0.001 must be added for each 3°C. above or deducted for each 3°C. below.
5. If the quantity of urine is small and the specific gravity is important, the urine may be diluted with distilled water and the specific gravity read; to obtain the correct number, multiply the last two figures of the specific gravity number by the amount of dilution. *This diluted urine cannot be used for qualitative or quantitative tests.*

B. ***Normal.***

1. For a 24 hour specimen—1.015 to 1.025. It varies inversely with the volume and directly with the amount of salt, urea, and protein in solution. For each gram of albumin per 100 cc. of urine, the specific gravity is increased 0.003.
2. Single specimens may range from 1.002 to 1.030.

C. ***Pathological Significance.***

1. Varies from 1.001 to 1.060.
2. Low in chronic nephritis and diabetes insipidus.
3. High in diabetes mellitus, fevers, and acute nephritis.
4. See Renal Function Tests, page 27.

IV. Reaction.

A. ***Nitrazine Paper.***

1. To determine pH of urine, use one of the following methods:
   a. With a clean glass rod transfer a drop of urine to the surface of a piece of nitrazine paper and spread evenly by stroking or leave a small drop as such on the paper.
   b. Dip paper into urine three consecutive times and then shake off excess liquid.
2. One minute after placing urine on paper, compare color with color chart.

B. ***Litmus Paper.***

1. Test urine with neutral litmus paper dipping a strip of the paper into the urine.
   a. Pink color—acid.
   b. No change in color—neutral.
   c. Blue—alkaline.
2. When the test is alkaline, dry the litmus paper with heat. If it loses its blue color, the "alkalinity" is due to a volatile substance, ammonia; if a nonvolatile alkali the paper remains blue.

C. ***Normal Values.***

1. Freshly voided urine is usually acid in reaction, the pH ranging from 4.8 to 7.5 with an average of 6.
2. It may be alkaline after a full meal, after taking large quantities of citrus fruits, or as a result of taking alkalies.
3. Twenty-four hour specimens are less acid than freshly passed specimens and may become alkaline after standing due to the decomposition of urea by bacteria and the liberation of ammonia.

D. ***Pathological Significance.***

1. Acidity is increased in acidosis, fevers, and a diet with an excess of protein.
2. May be alkaline in chronic cystitis and urine retention due to decomposition of the urine in the bladder. Same reason as in C3.
3. Fixed alkaline urines are associated with anemia, rapid absorption of transudates, some nervous diseases, obstructing gastric ulcer, severe vomiting, and alkaline therapy.

V. Cloudiness.

A. **Report** as +, ++, +++, or ++++ after suspending the sediment in the urine by shaking. See Table 1 on page 3.

B. **Freshly voided urine** is usually clear; but is sometimes cloudy due to the following sediments which are identified with the microscope.

1. *Amorphous phosphates* form a white cloud or precipitate in neutral or alkaline urine. This cloud disappears upon addition of dilute acetic acid.
2. *Amorphous urates* form a white or pink cloud of sediment (brick dust deposit) in acid urine which disappears on heating.

3. *Epithelial cells and mucus* give cloudiness to urine when present in large amounts.
4. *Blood* gives urine a red or brown smoky color.
5. *Pus* makes urine turbid, but clears up on filtering or centrifuging.
6. *Bacteria* produce a uniform cloudiness which does not settle out and cannot be filtered out.
7. *Fat and chyle* may render urine turbid.
8. *Shreds* are often present in chronic gonorrhea.

VI. Odor.

A. ***Normal.***

1. Aromatic, due to volatile acids. More marked in concentrated urine.
2. Various articles of diet and drugs impart peculiar odors, especially asparagus and turpentine.

B. ***Abnormal.***

1. It is ammoniacal after decomposition; important only in fresh urine and found in cystitis and urine retention.
2. It is fruity in diabetes if ketone bodies are present.

Chemical Tests

I. Protein.

✓ **A.** ***Albumin*** in the urine is derived from a number of sources.

1. *Physiologic albuminuria* appears after excessive muscular exertion, prolonged cold baths, excessive ingestion of proteins, etc.
2. *Orthostatic or postural albuminuria* appears after a person has been in an erect position and disappears with rest in bed.
3. *Accidental or False Albuminuria.*
   a. Due to pus, blood, and vaginal discharge.
   b. Found in pyelitis, cystitis, and chronic vaginitis.
4. *Pathologic Albuminuria.*
   a. Albumin in kidney disease is derived from the blood plasma and indicates increased permeability of the glomerular filter. Albumin, because of its smaller molecule, is excreted in larger amounts than globulin or fibrin, markedly decreasing the albumin-globulin ratio in the blood plasma when albuminuria is of severe grade. See Table 4 on page 31.
   b. Also present in febrile diseases, toxemia of pregnancy, passive congestion of the kidneys, and anemias.

B. ***Principle of the Tests:*** All methods depend upon the precipitation of protein by chemical agents or coagulation by heat.

C. ***Qualitative Tests.***

1. *General Considerations.*
   a. Urine must be clear; if not filter or centrifuge.
   b. If the urine is alkaline, add acetic acid drop by drop until slightly acid.
2. *Robert's Test.*
   a. Place 3 to 5 cc. of clear urine in a test tube three-fourths to an inch in diameter.
   b. Place the tip of a 5 or 10 cc. volumetric pipette containing Robert's reagent to the bottom of the tube and allow about 3 cc. of the reagent to layer beneath the urine.
   c. If several tests are being done, wipe off the tip of the pipette before inserting it into the next tube.
   d. A positive test is indicated by a white ring at the zone of contact.
   e. The ring must be read within 3 minutes after adding the reagent and with the eyes on the level of the contact ring. In order to observe a faint ring create a dark background by placing a finger between the tube and source of light.
   f. Rings that are 1 to 2 mm. above the zone of contact are due to mucin and nucleoalbumin; rings 1 to 2 cm. above the zone of contact are due to urates, uric acid, urea, and bile acids. These are not to be reported positive for albumin.
   g. The test may be performed by holding a test tube containing a few cc. of Robert's reagent in an inclined position and allowing filtered urine to run slowly down the side of the tube from a pipette or medicine dropper. It is difficult to get a good contact ring this way.
   h. Record the result according to Table 1 on page 3 (ring test).
   i. *Robert's reagent:*

Saturated magnesium sulfate (U. S. P. $MgSO_4 \cdot 7H_2O$) sol. (add 1 liter of water to 800 gm.)	5 parts
Nitric acid (conc.)	1 part

3. *Heller's Nitric Acid Test.*
   a. Perform the test as directed under Robert's test using conc. nitric acid instead of Robert's reagent and read the white ring at the zone of contact in the same manner.
   b. A red or reddish-violet ring which tends to extend downward into the acid is sometimes obtained with normal urine, but this ring is due to the reaction of the urinary pigments with nitric acid and is below the white one produced by albumin.
   c. If bile is present, a play of colors (red, violet, blue, and green) will be found at the line of contact.

d. Interfering rings outlined under Robert's test also apply to the nitric acid test.

e. A less accurate but rapid method of performing a series of tests is as follows:

   1) Use a piece of glass tubing with an inside diameter of about 5 mm.
   2) Immerse this tube in the urine to about an inch, wipe off the outside, and immerse in a test tube containing 2 inches of conc. nitric acid.
   3) By holding the finger over the top until the upper level of the urine in the tube is just below the surface of the acid in the test tube and then removing the finger and lowering the tube to the bottom of the test tube, the nitric acid rises in the tube and forms a sharp line of demarcation between the two.
   4) Place the finger over the upper end of the tube while removing it, hold up to the light, and read the width of the white ring in terms of +, ++, etc.
   5) Rinse out the tube and fill with another urine, using the same test tube of nitric acid for a number of tests.

4. *Sulfosalicylic Acid Test* (Exton's Method).

a. Mix equal volumes of clear urine and Exton's reagent in a test tube.

b. If no cloudiness develops, albumin is absent.

c. If cloudiness appears, warm gently but do not boil.

d. If the cloudiness persists or increases on heating, albumin is present.

e. Read while warm and record the result according to Table 1 on page 3 (heat test).

f. Proteoses will cause a cloud on cooling.

g. Bence-Jones protein causes a heavy precipitate which clears partially or wholly upon boiling.

h. *Exton's qualitative reagent.*

Sodium sulfate, anhydrous	50 gm.
Sulfosalicylic acid	50 gm.
Distilled water to make 1 liter.	

   1) Dissolve the sodium sulfate in 800 cc. of water with the aid of heat.
   2) Cool, add the sulfosalicylic acid, and make up to volume with water.

5. *Heat and Acetic Acid Test.*

a. Fill a test tube three-fourths full of clear urine and gently heat the upper portion to boiling; boil for 1 or 2 minutes being careful not to shake the tube more than necessary. Rotate the tube to prevent cracking.

b. A turbidity is due either to phosphates, carbonates, or albumin.

c. Add 3 drops of 10% acetic acid drop by drop, boiling between each drop.

d. A white cloud now disappearing is due to earthy phosphates or carbonates.

e. A faint trace of albumin may appear only upon the addition of the acid. Larger traces appear upon boiling and may become heavier upon the addition of the acid.

f. The addition of too much acid may dissolve faint traces of albumin and give a falsely negative reaction.

g. In order to detect slight traces, the tube must be held against a black background.

h. See Table 1 on page 3 (heat test) for recording amount present.

6. *Purdy's Test Modified.*

a. Fill a test tube half full of filtered urine.

b. Add about one-fifth of its volume of saturated aqueous solution of sodium chloride to raise the specific gravity (high specific gravity prevents the precipitation of mucin).

c. Add 2 to 5 drops of glacial acetic acid.

d. Mix well and boil the upper portion gently. Rotate tube to prevent cracking.

e. A cloud denotes the presence of albumin.

f. Record amount present according to Table 1 on page 3 (heat test).

D. *Quantitative Tests.*

1. *Esbach's Test* (Tsuchiya's Modification).

a. The urine must be filtered until clear.

b. If the qualitative test for albumin is more than two plus, dilute one part urine with four parts of distilled water.

c. If not strongly acid, add 1 or 2 drops of glacial acetic acid.

d. Add enough powdered pumice or barium sulfate to an Esbach's tube to just cover the bottom of the tube. This increases the rate of sedimentation.

e. Fill tube with urine to mark U and add Tsuchiya's reagent (or Esbach's) to mark R.

f. Close tube with a rubber stopper and invert slowly 10 times.

g. Place in a test tube rack and keep in a cool place for sedimentation.

h. The height of the sediment must be read at the end of 30 minutes.

i. If pumice is not added, read at 24 hours.

j. The readings on the tube indicate grams of albumin per liter of urine. To change to per cent divide by 10.

k. If the urine has been diluted, multiply the final reading by the dilution.

1. *Tsuchiya's reagent.*

Phosphotungstic acid (crystals)	1.5 gm.
Alcohol (95%)	93.5 cc.
Hydrochloric acid (conc.)	5.0 cc.

Dissolve the phosphotungstic acid in the alcohol before adding the acid.

2. *Shevky and Stafford's Method.*
   - a. Test the urine for albumin; if over one plus, dilute 1 part urine with 9 parts of distilled water.
   - b. Place 8 cc. of urine (or diluted urine) in a 15 cc. graduated centrifuge tube. (Preferably calibrated tubes with long narrow tips.)
   - c. Carefully stratify 5 cc. of Tsuchiya's reagent over the urine.
   - d. Stopper and invert slowly 3 times.
   - e. Let stand exactly 1 minute after mixing; centrifuge for 15 minutes at 1800 revolutions per minute. (The greater the delay between mixing and centrifuging, the greater is the volume occupied by the precipitate.)
   - f. Read the volume of packed precipitate.
   - g. *Calculation:* With undiluted urine each 0.1 cc. of precipitated protein is equivalent to 0.036 gm. of protein per 100 cc. of urine.
3. *Kingsbury's Test (Life Insurance Method).*
   - a. Pipette 2.5 cc. of clear urine into a test tube and add 7.5 cc. of 3% sulfosalicylic acid.
   - b. Invert 3 times.
   - c. Allow to stand 10 minutes and then compare the turbidity with that of the permanent standards.
   - d. Record the value of the standard most closely matched.
   - e. For values above 100 mg. per 100 cc., repeat the test with urine diluted sufficiently with distilled water to come within the range of the standards; multiply the result by the dilution.
   - f. *Permanent standards.*
     - 1) They are the formazin standards of Kingsbury-Clark and may be obtained from Magar Chemicals, Inc., Cornwall Landing, New York.
     - 2) They can be made, but it is a time consuming procedure.

II. Bence-Jones Protein.

A. *General Considerations.*

1. Bence-Jones protein is thought to be a proteose and is usually associated with a high serum globulin.
2. It is found in about 50% of cases of multiple myeloma, and sometimes in other bone tumors, chronic leukemia, empyema, and hyperparathyroidism. It is found along with albumin in some cases of chronic nephritis with high blood pressure and edema; also in some apparently healthy young people with high blood pressure.

B. *Method.*

1. Fill a test tube three-fourths full of urine and gently heat in a beaker of water with a thermometer in it.
2. If this protein is present, the urine will begin to be turbid at about 40°C. and a precipitate will form at about 60°C.
3. Acidify very slightly with 10% acetic acid and bring to the boiling point; the precipitate wholly or partially dissolves.
4. Filter while hot, the Bence-Jones protein in the filtrate reappears on cooling to 60°C.
5. As a confirmatory test the protein may be precipitated by adding nitric acid at room temperature. This precipitate wholly or partially clears up on boiling and reappears on cooling. Filter immediately after boiling if albumin is present.

III. Mucin.

A. *General Considerations.*

1. Important chiefly because it may be mistaken for albumin in most of the tests.
2. Mucin and mucoid are glycoproteins and on boiling with an acid or alkali will reduce the copper sulfate in Benedict's reagent.
3. Found in increased amounts in irritations and inflammations of the mucous membrane of the urinary tract or the vagina.

B. *Detection.*

1. If the urine was positive for albumin, remove the albumin by boiling 5 cc. of the urine and filtering while hot.
2. When the filtered urine is cold, dilute 2 cc. with 6 cc. of distilled water to prevent the precipitation of urates.
3. Make strongly acid by adding a few drops of glacial acetic acid.
4. If mucin is present, the urine will become turbid.
5. The precipitated mucin will dissolve on the addition of a few drops of 10% sodium hydroxide.

IV. Glucose.

A. *Normally* present in 0.01 to 0.03 gram per 100 cc. of urine.

B. *Glycosuria.*

1. In diabetes mellitus sugar usually appears in the urine when the level of glucose in the

blood rises above 160 mg. per 100 cc. This is the renal threshold (varies from 114 to 216 mg.) for glucose for most people.

2. In renal diabetes glucose appears in the urine with normal or low blood levels.
3. Glycosuria may be present after brain injury and after coronary thrombosis.

C. ***General Considerations.***

1. Albumin, when present in appreciable amounts, must be removed. The presence of albumin interferes with sugar reactions. Add a few drops of 10% acetic acid to a few cc. of urine, boil, and filter.
2. When sugar is found, always test for acetone and diacetic acid.
3. False positive tests may be due to homogentisic acid in alkaptonuria; increased ascorbic acid after ether anesthesia; formalin used as a preservative; also glycuronates from camphor, chloral hydrate, or morphine therapy and urates reduce copper slightly.

D. ***Qualitative Tests.*** (Single specimens may be used.)

1. *Benedict's Test.*

Principle: An alkaline copper sulfate reagent is reduced to cuprous oxide by the action of glucose and other reducing sugars.

a. Place 5 cc. of Benedict's qualitative reagent in a test tube.
b. Add 8 drops (0.5 cc.) of urine (no more), mix by shaking, and boil vigorously for 2 minutes over a flame. (Four drops of urine to 2.5 cc. of Benedict's reagent works just as well and saves Benedict's reagent.)
c. When doing a number of urines, the test tubes may be placed in a beaker of boiling water for exactly 5 minutes.
d. The tubes are read at once.
e. In the presence of glucose, the entire solution will be filled with a precipitate ranging in color from yellowish-green to red Normally a small quantity (up to 0.5%) of reducing substances are present, which appears as a slight yellow precipitate only when the reagent is cold.
f. Record the results according to Table 1 on page 3.
g. The reaction may be read by the amount of sediment after the tubes are allowed to cool slowly.
h. *Benedict's qualitative reagent.*

Copper sulfate (pure crystalline $CuSO_4 \cdot 5H_2O$)	17.3 gm.
Sodium citrate, c.p.	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.

(or 200 gm. of crystalline $Na_2CO_3 \cdot 10H_2O$)
Distilled water to make one liter.

1) Dissolve the citrate and carbonate in 600 cc. of water with the aid of heat and filter.
2) Dissolve the copper sulfate in 100 cc. of water with the aid of heat and pour slowly into the first solution, stirring constantly.
3) Cool and make up to 1 liter.
4) This reagent keeps indefinitely.
5) It cannot be used for quantitative tests.

2. *Phenylhydrazine Test.*

Principle: Each sugar forms an osazone with a definite crystalline form when brought into contact with phenylhydrazine and acetate.

a. Place 0.1 gm. of pure phenylhydrazine hydrochloride in a wide test tube with 3 cc. of urine.
b. Add 0.2 gm. sodium acetate.
c. Heat in a boiling water bath from 30 to 60 minutes.
d. The annoying bumping can be reduced or obviated by shaking continually or by placing in the test tube a number of pieces of glass tubing varying in length from 1 to 3 inches so as to produce an organ pipe effect.
e. Set aside to cool or, if the glass tubes were used, pour the fluid into another hot test tube and allow to cool.
f. Examine the sediment with the microscope, using a 16 mm. objective.
g. If glucose is present, there will be yellow, needle-like phenylglucosazone crystals arranged mostly in clusters.
h. When traces of glucose are present, the crystals may not appear for 30 minutes or more.
i. The best crystals are obtained when the fluid is cooled very slowly and not agitated during cooling.
j. Glucose and fructose form the same osazone, see Fig. 1A.

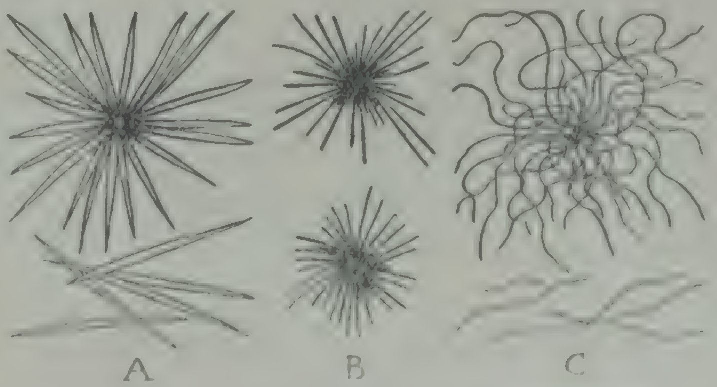


FIG. 1. Osazone crystals. A. Glucosazone. B. Lactosazone. C. Pentosazone.

k. Lactosazone crystals appear as delicate threads in clusters, resembling soft "pompons," see Fig. 1B.

l. Pentosazone crystals resemble a mass of tangled rootlets, see Fig. 1C.

3. *Fermentation Method.*

Principle: Glucose is fermented by yeast with the evolution of CO_2; lactose and pentose are not.

a. Boil about 50 cc. of urine to kill the bacteria.

b. Cool and mix about 25 cc. of the urine with one-quarter cake of fresh yeast.

c. Label 3 fermentation tubes A, B, and C.
   1) Fill the long arm of tube A and about half the bulb with the above mixture of yeast and urine. Remove all bubbles by tipping the tube with the thumb over the opening.
   2) Fill tube B in the same manner with a mixture of yeast and distilled water (control to see if yeast produces gas in the absence of glucose).
   3) Fill tube C in the same manner with a mixture of yeast and a solution containing glucose (control to see if yeast will produce gas in the presence of glucose).

d. Plug all tubes with cotton and place in a 37°C. incubator for 2 to 3 hours or leave standing at room temperature for 24 hours.

e. If fermentation tubes are not available, the following may be used.
   1) Invert a small tube (3/8 by 3 in.) in a large tube (3/4 by 6 in.) filled with the yeast and urine mixture. Label A.
   2) Place the thumb over the opening of the large tube and invert, turning the tube back slowly so that the small tube is filled completely.
   3) Set up two more sets of tubes, label B and C, using same mixtures as in fermentation tubes B and C above.

f. *Results:*
   1) If tubes A and C contain gas and tube B does not, the urine is positive for glucose.
   2) If tube B contains gas, the test must be repeated with different yeast.
   3) If tube C does not contain gas, the test must be repeated with different yeast.

E. *Quantitative Tests.* (Only samples of well-mixed 24 hour specimens are used.)

1. *Benedict's Method.*

Principle: Glucose (and other reducing substances) reduce the copper hydroxide to cuprous oxide which in the presence of potassium thiocyanate is changed to white copper thiocyanate.

a. Place 25 cc. of Benedict's quantitative reagent in a 250 cc. evaporating dish.

b. Add 5 to 10 gm. of anhydrous sodium carbonate and a few grams of powdered pumice stone or talcum to prevent bumping.

c. Heat to boiling and add the urine rather rapidly from a burette until a chalk white precipitate forms and the blue color begins to fade.

d. After this point is reached, add the urine drop by drop until the last trace of blue just disappears and a gray color remains. Half-minute intervals must be allowed to elapse between additions of urine in the final steps of the titration.

e. During the whole titration the mixture must be kept boiling vigorously and the loss of fluid by evaporation must be made up by adding distilled water.

f. If less than 5 cc. of urine are required, make a 1-10 dilution with distilled water and repeat.

g. *Calculation:*

The quantity of urine (make correction if dilution is made) used contains 0.05 gram of glucose, since the Benedict's solution is of such strength that 25 cc. is completely reduced by this amount of glucose. Using this figure, the number of grams in 24 hours or the number of grams per 100 cc. (percentage of sugar) can be computed.

   1) The percentage is obtained by dividing (0.05 × 100) or 5 by the number of cc. of urine required to reduce 25 cc. of Benedict's solution.

   2) To obtain grams in 24 hours, divide 0.05 by amount of urine needed to reduce 25 cc. of Benedict's solution and multiply by number of cc. of urine in the 24 hour specimen.

h. *Benedict's quantitative reagent.*

Copper sulfate (pure crystallized)	18.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.
(or 200 gm. of crystalline $Na_2CO_3 \cdot 10H_2O$)	
Sodium citrate, c.p.	200.0 gm.
Potassium sulfocyanate, c.p.	125.0 gm.
Potassium ferrocyanide solution (5%)	5.0 cc.
Distilled water to make 1 liter.	

   1) With the aid of heat, dissolve the carbonate, citrate, and sulfocyanate in about 600 cc. of water and filter.
   2) Dissolve the copper sulfate in 100 cc.

        of water with the aid of heat and pour slowly into the first solution, stirring constantly.
    3) Add the ferrocyanide solution, cool, and dilute to 1 liter.
    4) The copper sulfate should be weighed on an analytical balance.
    5) The solution keeps well.
    6) It should be checked by titrating with a standard glucose solution. Twenty-five cc. should be reduced by 5 cc. of the 1% stock glucose standard solution used in blood sugar determinations.

2. *Benedict's Test Tube Method.*
    - a. Pipette 5 cc. of Benedict's quantitative reagent into a large pyrex test tube.
    - b. Add 1 or 2 gm. of sodium carbonate and boil vigorously while agitating the tube gently.
    - c. From a 1 cc. pipette calibrated to 0.01 cc., add urine drop by drop to the solution, keeping the solution boiling while adding the urine.
    - d. When the blue color starts to fade, the solution should be boiled for 30 seconds between the addition of each drop.
    - e. The end-point is reached when the blue just disappears and a gray color remains.
    - f. If less than 1 cc. of urine is required, make a 1-5 dilution with distilled water and repeat.
    - g. *Calculation:*
        1) The number of cc. of urine used divided into (0.01 × 100) or 1 equals the glucose present in per cent since 0.01 gm. of glucose reduces 5 cc. of Benedict's reagent.
        2) For grams per 24 hours, divide cc. of urine used into total volume of urine and divide by 100.

V. Lactose.

. *General Considerations.*

. Often present in urine of pregnant women or during period of lactation.

. Lactose forms crystals with phenylhydrazine and is not fermented by yeast.

. *Rubner's Test Modified.*

. Place 3 cc. of urine in a test tube, add 2 cc. of ammonium hydroxide and 3 drops of 10% sodium hydroxide.

. Heat in a beaker of boiling water and observe after heating 2, 3, 4, and 5 minutes.

. A distinct reddish but not brilliant color is positive.

. Heating too long diminishes the color and a brownish tinge may appear.

. Other sugars give a yellow color.

VI. Pentose.

A. *General Considerations.*

1. May be present after ingestion of large quantities of pentose-rich substances (cherries, plums, fruit juices) or in habitual use of morphine.
2. There is a chronic form of pentosuria which is most commonly found in the Hebrew race.
3. Pentose forms crystals with phenylhydrazine and is not fermented by yeast.

B. *Tauber's Test.*

1. Place 1 to 2 cc. of a saturated solution of benzidine in glacial acetic acid (1 gm. in 25 cc.) in a test tube.
2. Add an equal amount of urine.
3. Place in a boiling water bath and watch for a cherry red color given by a pentose.
4. After 9 to 10 minutes the red will change to brown which is the color given by other sugars.

VII. Ketone Bodies.

A. **Ketone bodies** are acetone, diacetic acid, and beta-hydroxybutyric acid. Their chief source is faulty catabolism of fats whereby the fatty acids are not completely oxidized. When these are present in the urine, there is a state of ketosis.

B. **Ketosis** occurs most frequently in diabetes mellitus, but is also found in starvation, Von Gierke's disease, eclampsia, fevers, certain nervous disorders, after prolonged vomiting or diarrhea, and after ether or chloroform anesthesia.

C. *General Considerations.*

1. Specimens positive for sugar and all specimens from prenatal patients and patients in acidosis must be examined routinely for acetone and diacetic acid.
2. If the Rothera test, which is for both acetone and diacetic acid, is positive, the Gerhardt test for diacetic acid should be made. This is a rough quantitative method to determine the degree of ketosis as the Rothera test is a much more sensitive test than the Gerhardt test. See methods for the sensitivity of the tests.
3. The tests should be made within 3 hours, preferably immediately after collection of the urine. If this cannot be done, the specimen should be kept in the refrigerator.
4. Fresh urine contains relatively little acetone, but on standing the diacetic acid is decomposed to acetone.
5. The diacetic acid excreted in the urine in a given time is approximately the same whether the volume is large or small; it is therefore

necessary to take into account the volume of urine excreted in a given time.

D. *Rothera's Test for Acetone and Diacetic Acid.* ***Principle:*** Sodium nitroprusside (sodium nitroferricyanide) is decomposed to $Na_4Fe(CN_6)$, $NaNO_2$, and $Fe(OH)_3$ in an alkaline solution. These chemicals are strong oxidizing agents and in the presence of diacetic acid and acetone yield a complex having a rose or purple color. The ammonium sulfate acts as a buffer maintaining the alkalinity within the range at which the complex has a purple color. The test is 15 to 20 times more sensitive for diacetic acid than for acetone.

1. To 5 cc. of fresh urine, add ammonium sulfate crystals until saturated (about 1 gram).
2. Add 2 drops of sodium nitroprusside reagent and mix thoroughly.
3. Overlay with ammonium hydroxide.
4. If acetone is present, a red to purple color will develop at the line of contact. The color may not appear for 10 to 15 minutes.
5. The ring tends to be more red than purple in low concentrations. A definite purple color appears with 2 mg. per cent of diacetic acid (expressed as acetone) and 30 mg. per cent of acetone.
6. Urine collected after a heavy meal may give a purplish or violet color within 30 seconds which fades within 3 to 4 minutes; this is not a positive test.
7. Amorphous urates may give a brown or orange color.
8. *Sodium Nitroprusside Reagent.*

Sodium nitroprusside (nitroferricyanide) crystals	10 gm.
Sulfuric acid (conc.)	2 cc.
Distilled water to make	100 cc.

Keep in a brown bottle.

E. *Gerhardt's Test for Diacetic Acid.*

1. To 5 cc. of fresh urine in a test tube, add 10% ferric chloride solution drop by drop until all the phosphates are precipitated.
2. Filter, if necessary, and add more ferric chloride.
3. If a red wine color appears, it merely indicates the possible presence of diacetic acid, as salicylates, phenol, antipyrine, and sodium bicarbonate give a similar color.
4. If this red color is obtained, the test must be repeated on a fresh portion of the urine as follows:
   a. To about 5 cc. of urine in a test tube, add an equal volume of water and 1 drop of nitric acid and boil down to the original volume.
   b. Cool and add the ferric chloride as before.
   c. Boiling drives off the diacetic acid, so if there is no red color in this second test, diacetic acid was present in the urine and the test is positive for diacetic acid.
5. This test is positive in a concentration of 100 mg. per cent of diacetic acid (expressed as acetone).

VIII. Bile Pigment.

A. *General Considerations.*

1. Bilirubin is not normally found in the urine but may be demonstrable when there is no visible jaundice of the skin or sclera.
2. In complete obstructive jaundice there is bilirubin without urobilinogen in the urine.
3. In partial obstruction and hepatogenous jaundice, both bilirubin and urobilinogen may be present in the urine.
4. In hemolytic jaundice urobilinogen is present, but no bilirubin unless there is liver damage.

B. *Gmelin's Test* (Modified).

Principle: After precipitation of the bile pigment by barium chloride, it is oxidized by acids to derivatives like biliverdin (green), bilicyanine (blue), and choletelin (yellow).

1. Add 10 cc. of a 10% solution of barium chloride to 20 cc. of urine, mix, and let stand a few minutes.
2. Filter through a small filter paper.
3. Spread filter paper on a dry piece of filter paper and add 1 or 2 drops of yellow nitric acid to the center of the paper.
4. A positive reaction is indicated by a play of colors; green on the periphery, then in order toward the center, blue, violet, red, and yellow. The absence of green excludes the presence of bile pigment.
5. Yellow nitric acid can be made by adding a small piece of pine wood (applicator stick) to the acid.

C. *Harrison's Spot Test.*

Principle: Same as for Gmelin's test.

1. Add 10 cc. of a 10% solution of barium chloride to 20 cc. of urine, mix, and let stand a few minutes.
2. Filter through a small filter paper.
3. Spread filter paper on a dry piece of filter paper and add 1 or 2 drops of Fouchet's reagent.
4. A positive reaction is indicated by a blue to green color.
5. *Fouchet's Reagent.*

Trichloroacetic acid	25 gm.
Distilled water	100 cc.
Ferric chloride solution (10%)	10 cc.

D. *Methylene Blue Test.*

1. Add a 0.2% aqueous solution of methylene blue drop by drop to 5 cc. of a pre-breakfast urine. The urine will first turn green.
2. The number of drops needed to change the color from green to blue is the end-point.
3. More than 4 drops is considered a positive test for bilirubin; 4 drops or less is a negative test.
4. If more than 8 drops are required, make a 1 to 5 dilution of the urine and repeat. Multiply the number of drops by the dilution.
5. An occasional false positive result may be obtained with urine having a high specific gravity.
6. Urine obtained after large doses of penicillin, riboflavin, or quinacrine (atabrine) may give false positive results.

E. *Foam Test.*

1. Shake urine; if foam on top is yellow, bile may be present.
2. Certain drugs (pyridium, serenium) and increased amounts of urobilin compounds will give a false positive test.

IX. Urobilinogen.

A. *General Considerations.*

1. Urobilinogen is formed in the intestine by the action of bacteria on bile pigment. Part is excreted in the feces, part absorbed by the intestine and returned to the liver by way of the portal vein to be reconverted to bilirubin, and a small amount normally passes through the liver and is excreted by the kidneys.
2. If no bile passes into the intestine, as in complete obstruction of the bile ducts, no urobilinogen will be formed and none will be found in the urine.
3. In all conditions with excessive destruction of the erythrocytes, there will be an increase of bile pigment in the intestine resulting in an increased formation and absorption of urobilinogen. The liver can not reconvert all of this urobilinogen into bile so there is an increased excretion of urobilinogen in the urine.
4. In partial damage to the parenchyma of the liver, there is an increase of urobilinogen in the urine because the damaged liver cells are unable to remove it from the blood at the normal rate for reconversion into bilirubin.
5. A temporary increase of urobilinogen in the urine may be caused by constipation.

B. *Wallace-Diamond Test.*

Principle: The test depends upon the reaction between urobilinogen and paradimethylaminobenzaldehyde to form a cherry-red compound.

1. The test should be made soon after the urine is voided since urobilinogen is rapidly oxidized to bilirubin on exposure to air.
2. Place 10 cc. of urine in a test tube 15 mm. in diameter. The urine must not be cold.
3. Add 1 cc. of modified Ehrlich's reagent and mix by inverting the tube several times.
4. Let stand 5 minutes.
5. A cherry-red color appears if urobilinogen is present in abnormal amount. A light red and shades of pink appear when it is present in normal quantity. The examination for color should be made by viewing the contents through the mouth of the tube, holding it at a slight angle over white paper.
6. If positive, dilutions of the urine, 1-10, 1-20, 1-30, etc., should be made with slightly warmed distilled water and the test repeated. A slight color in the 1-20 dilution is normal.
7. Report dilution which gives a light red color.
8. The test is of no value in infections of the urinary tract due to the interference of nitrites produced by bacteria.
9. Formalin interferes with the test.
10. Urines containing bile and nitrites turn green when Ehrlich's reagent is added.
11. Sulfonamides and novocaine give a greenish yellow color.
12. Pyridium, indole, and porphobilinogen give false positive reactions.
13. *Ehrlich's Reagent (Modified).*

Distilled water	100 cc.
Hydrochloric acid (conc.)	150 cc.
P-dimethylaminobenzaldehyde	0.7 gm.

C. *Quantitative Urobilinogen.*

1. A 24 hour specimen must be used for an accurate quantitative determination.
2. Collect urine in a brown bottle containing 100 cc. of benzin (petroleum ether) and 5 gm. of anhydrous sodium carbonate.
3. See method (p. 116) in Section on Liver Function Tests.

X. Porphyrins.

A. *Coproporphyrin.*

1. Type I is increased in vitamin deficiency (pellagra), liver damage, and acute porphyria (type sensitive to light).
2. Type III is increased in toxic states, such as lead poisoning, after salvarsan, quinine and sulfonamide therapy, in acute intermittent porphyria, and in acute poliomyelitis.
3. It may be identified by the absorption spectra.

B. ***Uroporphyrin.***

1. Type I is the predominate porphyrin increased in acute porphyria.
2. Type III is the predominate porphyrin increased in acute intermittent porphyria.
3. If present in large amounts, it gives a port-wine color to the urine or the urine may become red only after standing.

C. ***Porphobilinogen.***

1. Found in acute intermittent porphyria.
2. It is a precursor of uroporphyrin III and is rapidly converted to uroporphyrin when the urine is acidified to 1 per cent with hydrochloric acid and left at room temperature overnight.
3. *Method* (Watson and Schwartz's).
    - a. The urine must be a freshly voided specimen.
    - b. Acidify with 10% HCl until a pH between 3.5 and 5 (important). Nitrazine paper may be used as an indicator.
    - c. Mix equal parts of urine and modified Ehrlich's reagent in a test tube.
    - d. If a red color is obtained, add an equal volume of a saturated aqueous solution of sodium acetate.
    - e. Add a few cc. of chloroform and mix thoroughly.
    - f. The red aldehyde compound of porphobilinogen remains in the aqueous fraction, while that of urobilinogen or indole is completely extracted by the chloroform.
    - g. The test must be carried out with these exact proportions of the reagents.
    - h. For a rough quantitative estimation make serial dilutions of the urine (1-10, 1-20, 1-40, etc.) and repeat the test.

XI. Phenylpyruvic Acid.

A. ***General Considerations.***

1. Phenylpyruvic acid is formed by oxidative deamination of phenylalanine.
2. It is increased in phenylpyruvic oligophrenia and thiamin deficiency.

B. ***Method.***

1. Add 2 to 3 drops of approximately 5 N sulfuric acid (25%) to 10 cc. of urine.
2. Add 3 to 5 drops of 10% ferric ammonium sulfate.
3. A green color indicates the presence of an abnormal amount of phenylpyruvic acid.

XII. Blood.

A. **Blood** may be in the form of intact erythrocytes (hematuria) or hemoglobin (hemoglobinuria).

1. *Hematuria* occurs in acute nephritis, passive congestion of the kidneys, calculi, malignant papilloma, renal carcinoma, tuberculosis of the kidney, chronic infections, after administration of sulfonamides and certain other drugs, and as a result of hemolytic poisons.
2. *Hemoglobinuria* appears after hemolytic poisons, severe burns, black water fever, paroxysmal hemoglobinuria, hemolytic transfusion reactions, and sulfonamide anemias. When free hemoglobin reaches an average of 150 mg. per 100 cc. of plasma (30-300 mg.), it is excreted by the kidneys.
3. The benzidine test is positive with hemoglobin, methemoglobin, carbon monoxide hemoglobin, and acid hematin but not hematoporphyrin.

B. ***Benzidine Test.***

Principle: The peroxidase activity of hemoglobin decomposes hydrogen peroxide and the liberated oxygen oxidizes the benzidine.

1. *General Considerations.*
    - a. If only a few blood cells are present, the test should be made on the sediment from a centrifuged specimen. It will be positive when there are twelve or more erythrocytes per high power field.
    - b. Pus will produce a blue color but not if the urine is previously boiled.
    - c. Benzidine labeled for blood test must be used.
    - d. Too much benzidine or too much hydrogen peroxide interferes with the delicacy of the test.
    - e. If in doubt as to the freshness of hydrogen peroxide, add a few drops of 10% potassium dichromate solution and a few drops of conc. sulfuric acid to 2 cc. of peroxide. A blue color indicates it is active.
    - f. A false positive benzidine reaction is obtained when the test is done in a test tube which has cuprous oxide left in it from a positive sugar reaction.
        - 1) An easy way to remove the copper film is to rinse out the tube with the final solution left from a Folin-Wu blood sugar test.
        - 2) There is enough phosphomolybdic acid in the solution to reduce the cuprous oxide in the tubes.
    - g. Bromides, iodides, nitric acid, and formalin will also give a false positive reaction.
    - h. Fats give false positive reactions and if present should be removed by extracting with ether (see confirmatory test page 102).
    - i. Ascorbic acid may produce a false negative reaction.

2. *Method.*
   - a. Place a pocket-knife point full of benzidine in a test tube.
   - b. Add 3 cc. of glacial acetic acid and shake until the acetic acid is thoroughly saturated. If necessary, add more benzidine.
   - c. Allow to settle and pour the clear supernatant liquid into another clean test tube.
   - d. Add 2 cc. of urine (previously boiled and cooled) to the clear benzidine solution and mix.
   - e. Add 1 cc. of fresh hydrogen peroxide and mix.
   - f. The appearance of a green or blue color within 5 minutes indicates the presence of blood. Report as follows:

Trace = faint green
+ = green
+ + = greenish blue
+ + + = blue
+ + + + = deep blue

   - g. A saturated solution of benzidine in glacial acetic acid (4 gm. to 100 cc.) keeps 2 weeks in a dark brown bottle.

C. *Guaiac Test.*

1. Place 4 cc. of urine in a test tube, add a few drops of glacial acetic acid, and mix.
2. In another test tube place a pocket-knife point full of powdered guaiac, add 2 cc. of 95% alcohol, and mix.
3. Add 2 cc. of fresh hydrogen peroxide, mix, and pour slowly down the side of the tube containing the urine so that it forms a layer on top of the urine.
4. If blood is present, a green to blue color will appear at the zone of contact of the two layers. The intensity of the color will vary with the amount of blood present, see benzidine test for method of reporting results.
5. A 1:25 solution of guaiac in alcohol keeps 8 months in a dark brown bottle.
6. False reactions are due to the same substances as in the benzidine test and to iron.
7. This test is less sensitive than the benzidine test.

XIII. Indican.

A. *General Considerations.*

1. Indole is the product of putrefaction of protein in the intestinal tract. It is absorbed and oxidized to indoxyl in the liver. The indoxyl combines with sulfuric acid and potassium to form indoxyl potassium sulfate or indican.
2. It is increased:
   - a. After diets rich in proteins.
   - b. In obstruction of the small intestine, in intestinal indigestion, cholera, and typhoid fever.
   - c. In diseases of the stomach associated with decreased hydrochloric acid, gastritis, and gastric cancer.
   - d. In diminished bile flow.
   - e. From decomposition of protein in other parts of the body as occurs in peritonitis, empyema, and large abscesses.

B. *Obermayer's Test.*

Principle: Obermayer's reagent will oxidize indican (indoxyl potassium sulfate) to indigo-blue or occasionally to indigo-red if the oxidization is slow.

1. To 3 cc. of urine in a test tube, add an equal volume of Obermayer's reagent and mix thoroughly.
2. Heat until the tube is warm.
3. Add 2 cc. of chloroform and mix by inverting several times, but avoid violent shaking.
4. Allow the chloroform to settle.
5. The indican in normal urine may give a faint blue color to the chloroform.
6. If present in excess, the color ranges from a trace to a very deep blue, depending upon the amount present. Occasionally a red color appears due to indigo-red.
7. Iodides and thymol give a reddish-violet color with this reagent which disappears on adding a crystal of sodium thiosulfate.
8. The presence of urotropin, formalin, and bile pigments interferes with the test. Bile pigments may be removed from the urine by adding one-fifth volume of 10% barium chloride, mixing, then filtering.
9. *Obermayer's Reagent.*

Hydrochloric acid (conc.)	1000 cc.
Ferric chloride	2 gm.

XIV. Diazo Substances.

A. *General Considerations.*

1. Certain unknown substances give a positive diazo reaction when treated with Ehrlich's diazo reagent and ammonium hydroxide.
2. These substances are found in typhoid fever, measles, and advanced tuberculosis.

B. *Method.*

1. For Ehrlich's diazo reagent see Van den Bergh test (p. 111). Mix 10 cc. of solution A with 0.1 cc. of solution B.
2. Mix 2 cc. of urine with 2 cc. of diazo reagent and shake vigorously.
3. Overlay with 2 cc. of ammonium hydroxide.
4. A bright pink to red color (not yellow or orange) at the zone of contact and particularly in the foam indicates a positive reaction.

XV. Bromides and Iodides.

A. ***Qualitative Test.***

1. Acidify about 10 cc. of urine with dilute sulfuric acid.
2. Add a few drops of fuming nitric acid, a few cc. of chloroform, and shake.
3. The chloroform, which settles to the bottom, assumes a yellow color in the presence of bromides.
4. Iodides give a pink to reddish-violet color to the chloroform.

B. ***Quantitative Test for Bromides.***

1. See Wuth's method in the Section on Chemistry (p. 325).
2. Iodides form a brown precipitate with gold chloride.
3. The excretion of bromides is quite constant and rapid after bromide therapy.

XVI. Fat and Chyle.

A. ***Fat.***

1. Shake 10 cc. of urine with 10 cc. of ether.
2. If fat is present, the urine becomes clear because the ether dissolves the fat.
3. Filter the ether through a small filter paper and let the paper dry.
4. If fat was present in the urine, the paper will be greasy.
5. Found in fatty degeneration of the kidney.

B. ***Chyle.***

1. The urine appears milky.
2. Chyle gives the same test for fat as described above.
3. It may be differentiated from ordinary fat by the fact that the particles are too small to be seen with the microscope, while fat globules may be easily seen.
4. Chyluria occurs most frequently in filariasis and is the result of obstruction and dilatation of the thoracic duct or its chyle-carrying tributaries followed by rupture of distended lymphatics into the urinary tract.

XVII. Total Solids.

A. **Total solids** may be roughly estimated by the following formula:

$S = 0.00233 \times g \times u.$
S = grams of solids in the urine.
g = the last two figures in the specific gravity.
u = cc. of urine in 24 hours.

B. ***Normal Value:*** About 60 grams in 24 hours.

XVIII. Congo Red Test for Amyloidosis or Nephrosis.

A. ***General Considerations.***

1. Congo red normally disappears very slowly from the blood plasma.
2. In amyloidosis it disappears more rapidly due to the affinity of amyloid substance for Congo red.
3. In nephrosis the dye is excreted in the urine more rapidly than normal, thus reducing the concentration in the plasma faster than normal but not as rapidly as in amyloidosis.

B. ***Paunz's Test.***

1. Test must be conducted in the morning before breakfast.
2. Inject intravenously 1 cc. of a sterile 1.5% solution of Congo red per 15 pounds of body weight or 1 cc. of a 1% solution per 10 pounds of body weight.
3. Four minutes later withdraw 10 cc. of blood from the opposite arm and place in a bottle containing 10 mg. of lithium oxalate and shake. Take precautions to prevent hemolysis.
4. One hour after the injection of the dye withdraw another 10 cc. of blood and place in a bottle containing oxalate.
5. After drawing the hour specimen of blood, collect a specimen of urine and note the color.
6. Centrifuge the bloods and remove the plasma.
7. Compare the 2 samples of plasma in a colorimeter, using the 4 minute sample as the standard.
8. Set the standard at 10 mm. and note the reading of the 1 hour sample.
9. Calculation:

$$\frac{\text{mm. 4 min. sample}}{\text{mm. 1 hr. sample}} \times 100 = \%\text{ dye remaining in plasma.}$$

$$100 - \%\text{ dye in plasma} = \%\text{ dye disappeared.}$$

C. ***Normal Value:*** Less than 40% of the dye disappears in 1 hour.

D. ***Pathological Findings.***

1. If over 60% disappears, amyloidosis is present.
2. If 40 to 60% disappears, amyloidosis or nephrosis is present.
3. In nephrosis more than a trace of dye is excreted in the urine.
4. Small amounts of amyloid may not decrease the dye by 40 per cent in 1 hour, therefore, a normal result does not exclude the diagnosis.

Analysis of Urinary Calculi

I. General Considerations.

A. ***Factors Influencing the Formation of Calculi.***

1. Alteration of protective colloids in the urine.
2. Supersaturation of "crystalline" constituents due to change in pH or to concentration of the urine.
3. Inflammation with clumps of bacteria, epithelial, and pus cells forming a nucleus for a stone.

4. Stasis of urine over a prolonged period of time.
5. Deficiency of vitamin A.
6. Excess of vitamin D.
7. Endocrine disorders, especially hyperparathyroidism.

B. *Nature of Calculi.*

1. They vary in size and shape according to their location.
2. They may be found in the renal pelvis, ureter, or bladder and are either passed in the urine or obtained by operation.
3. The simple form is made up of a single constituent.
4. The compound form contains two or more constituents which usually are arranged in concentric rings about a central nucleus.
    a. This is the most common form.
    b. Each layer and the core should be analyzed separately.

II. Description.

A. Examine stone carefully and then split with a fine saw so as to reveal the different structures within.

B. *Physical Characteristics.*

1. *Uric Acid Stones.*
    a. They often occur as multiple, smooth, round pebbles, which are devoid of luster and vary in size from a few to 30 mm. or more in diameter.
    b. Single stones frequently have a bumpy or eruptive type of surface, resembling miniature volcanic craters.
    c. Crushed stones are definitely yellow.
2. *Phosphate and Carbonate Stones.*
    a. They occur as compact balls (often several centimeters in diameter) or as large friable masses assuming the shape of the cavity where found.
    b. They resemble clay or chalk.
    c. Rarely do triple phosphate stones assume porous, coral-like formations, which suggest calcium oxalate except for their whiteness.
3. *Oxalate Stones.*
    a. They have a crystalline glint or smooth luster.
    b. Irregular, buff colored fragments may exhibit elaborate crystalline structures.
    c. The dark brown type is very compact and may take a grapeseed or berry-like form. Crushing produces a light-colored powder.
    d. The stones may be covered with a fine coral-like excrescence.
    e. Occasionally the whole structure is loose and porous.
4. *Cystine Stones.*
    a. They occur largely as pale yellow or white granules.
    b. They may resemble calcium oxalate in appearing to have a crystalline surface.
    c. Dissolve a small portion of the powdered stone in a drop of ammonium hydroxide (28%) and examine under the microscope for typical crystals.
5. *Sulfonamide Stones.*
    a. Sulfonamides may be deposited on the surface of uric acid stones.
    b. They may be mingled with phosphates and carbonates in a putty-like mass.
6. *Rare Stones.*
    a. Indigo calculi.
    b. Urostealith calculi are light colored and fatty.
    c. Cholesterol calculi are similar to cystine stones.
    d. Fibrin calculi are dark colored.

III. Analysis.

A. Follow Table 2. (See page 18.)

B. *Supplementary Tests.*

1. *Murexide Test.*
    a. Add several drops of concentrated nitric acid to a portion of pulverized stone in an evaporating dish and carefully evaporate to dryness on a water bath.
    b. Add 2 or 3 drops of concentrated ammonium hydroxide and observe for the following:
        1) *Uric acid*—deep yellow to orange-red or crimson becoming more purplish with ammonium hydroxide (bluish violet with sodium hydroxide).
        2) *Xanthine*—greenish yellow turning to orange with ammonium hydroxide becoming reddish on warming (deep orange-red with sodium hydroxide).
        3) *Protein*—pale yellow turning orange.
        4) *Sulfonamides*—yellow turning to mahogany brown.
2. *Ashing Process.*
    a. It is desirable to do this before testing for phosphates as given in Table 2.
    b. Place a portion of the pulverized stone in a porcelain crucible and ash.
    c. Cystine gives off a penetrating odor following the first curl of smoke and diminishes in intensity with increased heat.
    d. Cystine and urate stones blacken rapidly, the former burning "clean" and the latter with advancing greasy brown rings up the wall of the crucible; both leave the crucible empty.

TABLE 2. ANALYSIS OF URINARY CALCULI (Winer, J. H. and Mattice, M. R.)

Chemical Group	Reagents Added	Results
1. Urates and uric acid	Pulverized stone. 1 drop 20% Na_2CO_3. 2 drops uric acid reagent.	Prompt deep *blue color* (pale blue is negative).
2. Phosphates*	Pulverized stone. 4-5 drops ammonium molybdate solution (need excess of reagent).	Warm over flame to get distinct *yellow precipitate*, $(NH_4)_3PO_4 \cdot 12MoO_3$.
3. Oxalates	Pulverized stone. 2-3 drops 10% HCl—if no effervescence, cool and add pinch of MnO_2. Do not mix.	Tiny *bubbles* of gas "explosively" released from bottom.
4. Carbonates	Relatively large sample of pulverized stone. 8-10 drops 10% HCl.	Foaming *effervescence.*
Take up acid extract from 4 in aspirating pipette, the tip of which is lightly plugged with cotton. Remove cotton by seizing projecting wisp and divide "filtrate" into three aliquots for 5, 6, and **7.**		
5. Calcium*	Acid extract. 2-3 drops 20% NaOH.	Fine white precipitate or film from oxalate stones; dense precipitate from phosphate stones.
6. Magnesium	Acid extract. 2-3 drops of 20% NaOH. 2-3 drops reagent "M."	Reddish-purple reagent slowly becomes definitely *blue* (precipitate forms).
7. NH_4 Group	Acid extract. 2-3 drops 20% NaOH. 2-3 drops Nessler's solution. Alternative: pulverized stone and 2-3 drops of Nessler's solution.	*Yellowish orange* precipitate.
8. Sulfonamides	Pulverized stone. 2 drops 10% HCl (wait 30 seconds). 2 drops $NaNO_2$ (wait 30-60 seconds). 2 drops 0.5% ammonium sulfamate. 2-3 drops reagent "S."	*Brownish pink to magenta.*
9. Cystine	Pulverized stone. 1 drop NH_4OH; 1 drop NaCN (wait 5 minutes). 2-3 drops sodium nitroprusside.	*Beet-red* color; on standing may fade to orange red.

*Use a microscope slide for these tests, spot plate for the others. The artist's type is more satisfactory than the regular chemist's spot plate.

e. The residue from phosphate and oxalate stones is of about the same bulk as the original sample.
f. Phosphate ash is rarely white, while oxalate ash is more nearly white and effervesces if acid is added as it is converted to carbonate by heating.
g. Sulfonamides melt and form a black tarry mass which is resistant to ashing.

C. *Identification of Rare Stones.*

1. Place about 10 mg. of the powdered stone in a test tube and add 10 cc. of chloroform.
2. Stopper and shake.
3. Let stand until the insoluble material settles out, then decant the chloroform, saving both it and the residue.
4. A blue color in the chloroform indicates indigo.
5. Allow a few drops of the chloroform to evaporate on a fat-free glass slide and add a drop of Sudan III to the residue. Look for red fat droplets with the low power of a microscope; if present, they indicate urostealith.
6. To about 5 cc. of the chloroform, add 5 cc. of acetic anhydride and 0.5 cc. concentrated sulfuric acid. A green color indicates cholesterol.
7. Add 2 cc. of Millon's reagent to the residue and heat. A red precipitate is positive for fibrin.

IV. Reagents (may be kept in dropper-type bottles).

A. *Used for Tests in Table 2.*

1. *Sodium Carbonate*—a 20% solution.
2. *Uric Acid Reagent*—same as for blood uric acid determination (p. 284).

3. *Ammonium Molybdate*—dissolve 3.5 gm. of ammonium molybdate in 75 cc. of water and pour into 25 cc. of concentrated nitric acid.
4. *Hydrochloric Acid*—a 10% solution.
5. *Manganese Dioxide*—MnO_2.
6. *Sodium Hydroxide*—a 20 or 25% solution.
7. *Reagent "M"*—dissolve 1 mg. of p-nitrobenzeneazoresorcinol in 100 cc. of normal sodium hydroxide.
8. *Nessler's Solution*—same as used in blood analysis (p. 274).
9. *Sodium Nitrite.*
    a. A 0.1% solution keeps 2 weeks.
    b. Same as used in blood analysis.
10. *Ammonium Sulfamate*—a 0.5% solution.
11. *Reagent "S".*
    a. Dissolve 100 mg. of N-(1-naphthyl) ethylenediamine dihydrochloride in 100 cc. of water.
    b. Store in an amber bottle.
    c. Same as used in blood sulfonamide determination.
12. *Concentrated Ammonium Hydroxide*—a 28% solution.
13. *Sodium Cyanide*—prepare a 5% solution and preserve with 1 cc. of 28% concentrated ammonium hydroxide per 500 cc. of solution.
14. *Sodium Nitroprusside* (nitroferricyanide)—a 5% solution kept in a brown glass bottle and discarded when it shows signs of fading.

B. *Used in Supplementary Tests and in Identification of Rare Stones.*

1. *Concentrated Nitric Acid.*
2. *Chloroform.*
3. *Sudan III*—a saturated solution in 70% alcohol.
4. *Acetic Anhydride.*
5. *Concentrated Sulfuric Acid.*
6. *Millon's Reagent*—digest 1 part (by weight) of mercury with 2 parts (by weight) of nitric acid (sp. gr. 1.42) and dilute the resulting solution with 2 volumes of water.

Microscopic Examination of Urine Sediment

The microscopic examination of urine is of great importance and should never be omitted. When there is albumin, one should look very carefully for pus, blood, and casts. Examinations should be made on fresh specimens, as casts and erythrocytes disappear on standing. To keep for any length of time, add 1 drop of formalin to each ounce of urine.

I. Preparation and Examination of Sediment.

A. Normal urine as a rule contains very little sediment. There may be an occasional leukocyte, few epithelial cells, mucus, bacteria, and amorphous or crystalline forms of normal solids, such as amorphous urates and phosphates, uric acid crystals, calcium oxalate, and triple phosphate crystals.

B. *Centrifugation of Urine.*

1. Agitate the urine to stir up any sediment that may have settled to the bottom.
2. Alkaline specimens cloudy with phosphates may be slightly acidified with dilute acetic acid to redissolve the phosphates.
3. Highly acid specimens cloudy with urates may be slightly warmed to redissolve the urates.
4. Place 10 cc. of urine in a conical tipped centrifuge tube and centrifuge for 5 minutes at a low rate of speed (1800 r.p.m.).
5. Pour all of the urine out of the tube.
6. Place the tube in an upright position so the fluid remaining on the sides flows to the bottom to dilute the sediment.
7. Shake the sediment, place a drop on a glass slide, and cover with a cover glass. (If a case is to be followed from day to day, use the same capillary pipette to place the sediment on the slide each day.)
8. Examine under the microscope with the low and high power objectives.

C. *Examination of Sediment.*

1. Darken the field of the microscope by almost completely closing the diaphragm just beneath the stage.
2. A well-darkened field is absolutely necessary if hyaline casts are to be found because they are clear, colorless, and have a low refractivity.
3. First examine with the low power, then with the high power.
4. Record findings according to Table 1 on page 3.
5. *Errors to be avoided.*
    a. Careless transfer of sediment.
    b. Use of too much light in the microscopic examination.
    c. Use of high power only.
    d. Drying of sediment.
    e. Dirty equipment.
    f. Scratches on the cover glass or slide.

D. *Addis Sediment Count.*

1. Instruct the patient to take breakfast as usual, including fluids, but must abstain from all fluids (not food) until the urine has been collected the next morning.
2. The patient empties the bladder completely at 7 P.M. and discards the urine.

3. Save all urine until 7 A.M. the next morning and place in a clean receptacle containing 0.5 cc. of formalin. The bladder must be completely emptied at 7 A.M. and the specimen added to that in the receptacle.
4. In women the specimens must be collected by catheter.
5. Unless the pH of the urine is 6 or less, it is not satisfactory for this count because probably some casts may have been dissolved.
6. If urates are present, dissolve by immersing container in warm water.
7. Mix the specimens well by repeated inversion and measure the total amount accurately within 2 cc.
8. Transfer 10 cc. to a graduated centrifuge tube with a narrow tip and centrifuge 5 minutes at 1800 revolutions per minute.
9. Remove 9 cc. of the supernatant fluid and thoroughly mix the sediment in the remaining 1 cc. volume with a capillary pipette. If the sediment is heavy, a greater dilution may be made.
10. Transfer a drop of this sediment to a blood counting chamber with a leukocyte pipette in the same manner as though doing a leukocyte count.
11. The number and differential cast count is made under low power in all nine large squares (0.0009 cc. of urine).
12. The erythrocytes and leukocytes are counted in the same area under high power.
13. Repeat procedure 2 to 10 times and average the cells and casts counted.
14. *Calculation:*

$$N = \frac{s}{v} \times n \times \frac{V}{10}$$

V = volume of urine in cc. for 12 hrs.
10 = number of cc. of urine centrifuged.
s = volume in cc. of mixed sediment (1 cc.)
v = volume in cc. in which count was made (0.0009 cc.).
n = number of cells or casts counted.
N = number of cells or casts in 12 hour sample.

15. See Table 3 for normal values and findings in diffuse glomerulonephritis.

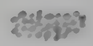
TABLE 3. NORMAL AND PATHOLOGICAL FINDINGS FOR THE ADDIS SEDIMENT COUNT

	Casts	Erythrocytes	Leukocytes
Normal	0–5,000	0–500,000	1,000,000
Acute nephritis	690,000	405,000,000	48,000,000
Chronic nephritis (active)	1,850,000	34,000,000	14,000,000
Chronic nephritis (latent)	48,000	16,000,000	2,400,000
Terminal nephritis	398,000	26,400,000	10,000,000

II. Unorganized Sediment.

A. *Amorphous Material.*

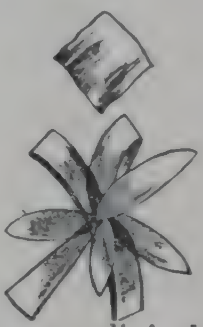
1. *Acid Urine.*
   a. *Amorphous urates of sodium, potassium,* sometimes magnesium and calcium appear as granules, usually brick red in color.



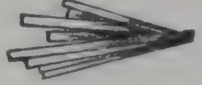
   b. They are dissolved by heat and by sodium hydroxide, but not by acetic acid.
   c. They have no clinical significance.
2. *Alkaline Urine.*
   a. *Amorphous phospates of calcium and magnesium* resemble amorphous urates but are colorless.
   b. They are dissolved in acetic acid, but not by heat.
   c. They are not important.

B. *Crystals in Normal Urine.*

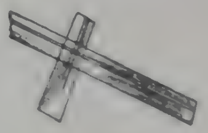
1. *Acid Reaction.*
   a. *Uric acid* — usually yellowish - brown, rhombic plates or rosettes; they may be colorless. They are not dissolved by heat, acetic acid, or hydrochloric acid and are soluble when heated with sodium hydroxide. They have no clinical significance except when they occur in fresh urine. With other symptoms they may serve as a warning of the possible danger of calculus formation.



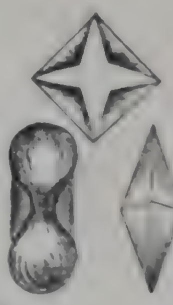
   b. *Sodium urate* — colorless or yellowish slender prisms arranged in fan- or sheaf-like structures.



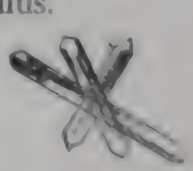
   c. *Calcium sulfate* — long, colorless needles or elongated prismatic tablets. Very rare and have no significance.



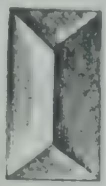
2. *Acid, sometimes neutral or slightly alkaline reaction.*
   a. *Calcium oxalate* — colorless, mostly "envelope" or dumbbell shaped, soluble in hydrochloric acid, insoluble in acetic acid. They are derived from various foods, notably spinach, rhubarb, and some berries. They suggest the possibility of a calculus.

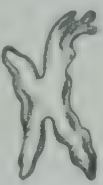


   b. *Hippuric acid* — colorless prisms or plates which may be so thin as to resemble needles, often conglomerated into masses. Soluble in acetic acid.

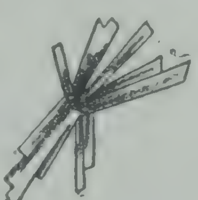


3. *Alkaline, sometimes neutral or slightly acid reaction.*
   a. *Ammonium-magnesium phosphate* (triple phosphate)—colorless and their usual form is some modification of the prism, with oblique ends, and occasionally take feathery or leaf-like forms. Most typical are the "coffin-lid" forms. In freshly passed urine they suggest stasis of urine in the kidney or bladder. They may also form calculi.

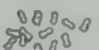




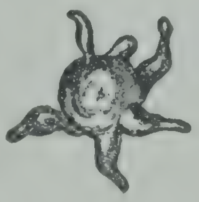
   b. *Dicalcium phosphate* — colorless prisms arranged in stars and rosettes. The individual prisms are usually slender, with one beveled, wedge-like end, but are sometimes needle-like. They are soluble in acetic acid.



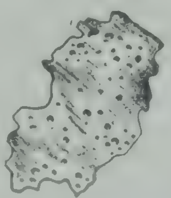
4. *Alkaline Reaction.*
   a. *Calcium carbonate* — colorless granules slightly larger than amorphous phosphates appearing singly or in masses and often appearing in dumb-bell forms. They dissolve in acetic acid with the evolution of gas.



   b. *Ammonium biurate*—yellow spherical bodies, usually with radial and concentric striations and bearing long prismatic spicules. They occur during ammoniacal fermentation and are only abnormal in freshly passed urine.

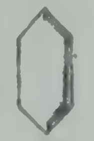


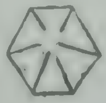
   c. *Calcium phosphate* — often forms large, thin, irregular, usually granular, colorless plates. Small plates might be mistaken for squamous epithelial cells. Sometimes called magnesium phosphate.



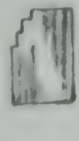
C. *Crystals Found in Acid Urine Indicating Abnormal Metabolism.*

1. *Cystine*—colorless, highly refractive, rather thick, hexagonal plates or quadrilateral prisms. They are soluble in hydrochloric acid but insoluble in acetic acid. The continued presence of these crystals in the urine is frequently associated with the presence of cystine calculi.

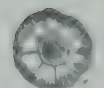




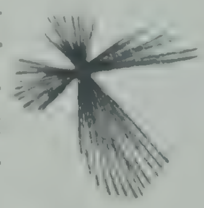
2. *Cholesterol*—large flat plates with one or more corners cut off. They may occur in nephritic conditions and lipoid nephrosis.



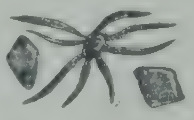
3. *Leucine*—highly refractive, yellow or brown spheres resembling fat globules, with delicate radiating and concentric striations. They are insoluble in ether or hydrochloric acid.



4. *Tyrosine* — colorless, fine needles, grouped in clusters or sheaves, crossing at various angles. Clusters may appear black in the center. Soluble in ammonium hydroxide and hydrochloric acid, but not in acetic acid. Leucine and tyrosine occur together in acute yellow atrophy and in other destructive diseases of the liver.



5. *Bilirubin and Hematoidin*—yellow or ruby red rhombic plates, needles, or granules. They are of little importance.

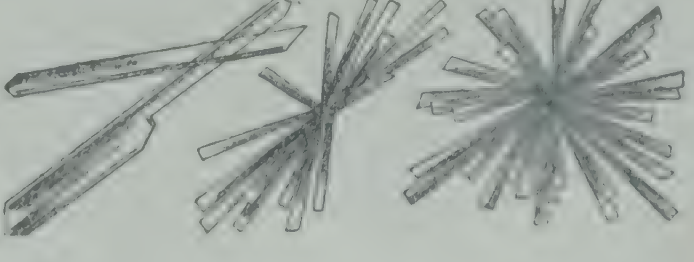


D. *Sulfonamide Crystals Found in Acid Urine.*

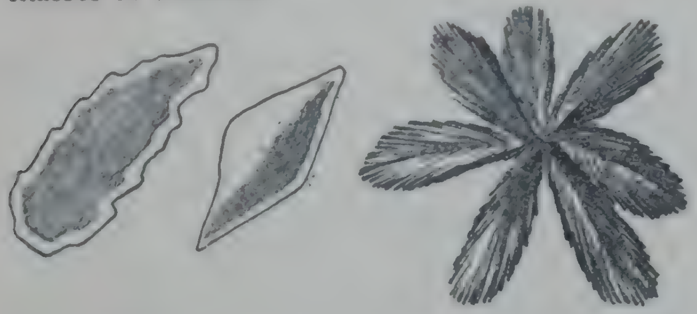
1. May cause kidney damage by blocking the tubules.
2. *Lignin Test for Sulfonamides.*
   a. *Principle:* Color development is due to the reaction of the arylamine group in the sulfonamide on crude cellulose (newspaper, paper towel, matchsticks, pine shavings) in the presence of acids.
   b. To be used when in doubt whether crystals belong to the sulfonamide group, or if there is a question whether the patient has received sulfonamide therapy. The test is positive 1 hour after the ingestion of a sulfonamide and remains positive up to 60 hours after the last dose.
   c. Place 1 or 2 drops of urine on a blank strip of newspaper or a paper towel.
   d. Add a small drop of 25% hydrochloric acid in the center of the moistened area.
   e. The immediate appearance of a yellow to orange color indicates the presence of a sulfonamide compound.

      + = yellow (roughly 0.10 mg.)
      + + = yellow to orange (10-30 mg.)
      + + + = orange (30-60 mg.)

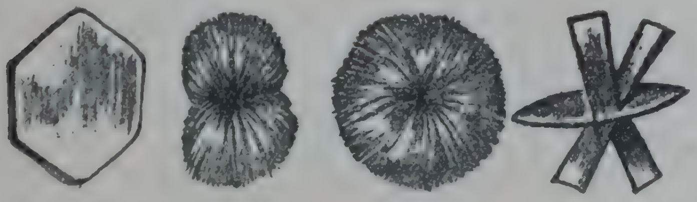
   f. Paper of bond quality or filter paper cannot be used.
3. *Sulfanilamide crystals* rarely appear in the urine. They are large colorless needles, frequently gathered together in sheaves or rosettes.



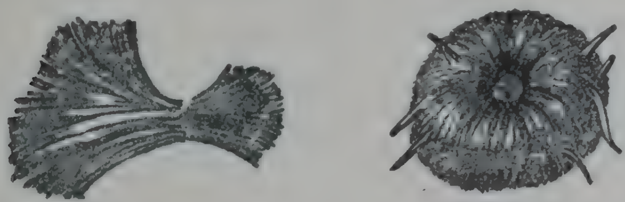
4. *Sulfapyridine crystals* are shaped like colorless arrowheads or whetstones; or they occur as brownish needles in large conglomerate masses or rosettes.



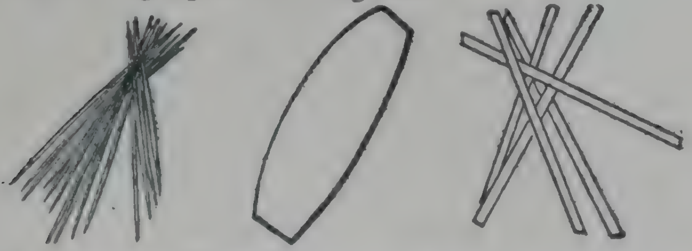
5. *Sulfathiazole crystals* appear as brownish shocks of wheat with central binding or as rosettes with radial striations; or they appear as colorless diamond and hexagonal shaped plates which are sometimes grouped in rosettes resembling uric acid crystals.



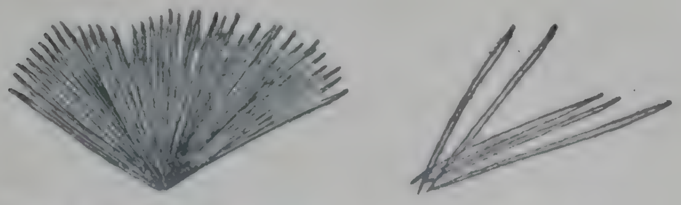
6. *Sulfadiazine crystals* are colorless to greenish brown, appear as shocks of wheat with eccentric binding and rosettes with radial striations, sometimes covered with needle-like processes (chestnut burr forms).



7. *Sulfaguanidine crystals* appear as colorless needles grouped as shocks of wheat with eccentric binding or rectangular plates with slight bulging in the long axis.



8. *Sulfamethylthiazole crystals* are colorless to greenish brown, appear as needles clumped in the shape of a fan.

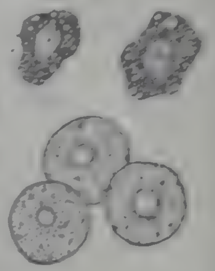


9. *Succinylsulfathiazole* is absorbed very slightly and is very soluble so that crystals do not usually appear in the urine.

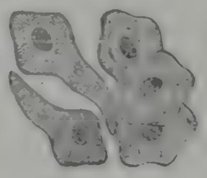
E. ***Fat*** appears as highly refractive globules of various sizes, which stain orange or red with Sudan III.

III. Organized Elements.

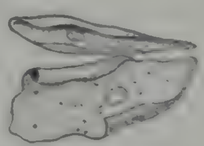
A. ***Epithelial Cells.***



1. *Small round epithelial cells* (renal cells) are mononuclear and slightly larger than leukocytes. They may come from the deeper layers of the lining of the urinary tract. If they are found on casts, their source is probably the uriniferous tubules; but if they are accompanied only by pus and but little albumin and especially if they adhere in shingle-like masses, they are probably from the pelvis of the kidney or from the bladder.
   a. They are abundant in glomerulonephritis, especially the acute form. They are nearly always fatty, most markedly in the chronic type.
   b. In nephrosis they may contain highly refractive lipoid granules.
   c. In hemolytic anemias and hemochromatosis, they may contain hemosiderin granules which can be stained by the Prussian blue reaction.
      1) Add a few drops of fresh reagent (equal parts of 2% potassium ferrocyanide and 1% HCl) to the sediment in the centrifuge tube.
      2) Let stand 10 minutes, recentrifuge, and examine the cells in the sediment.

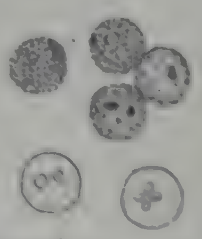


2. *Caudate and cylindrical cells* are 2 to 4 times the size of a pus cell and have various forms. They are for the most part derived from the transitional epithelium which lines the bladder, ureters, and pelves of the kidneys.



3. *Squamous cells* are large flat cells of the bladder or the superficial layers of the urethra or vagina. They are normally present in large numbers in the urine of women.

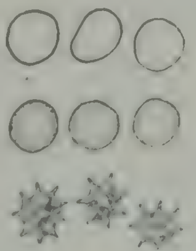
B. ***Leukocytes (Pus Cells).***



1. Under the microscope they have the same appearance as in a blood leukocyte count. To distinguish from erythrocytes add one drop of 10% acetic acid. The nuclei of pus cells become more distinct and erythrocytes dissolve.
2. In a strongly acid urine they are likely to be shrunken.

3. In alkaline urine they are swollen and have a tendency to form clumps.
4. Pus is an exudate containing cells and proteins. Therefore, urine containing large numbers of pus cells will give a positive test for albumin after the removal of the cells by centrifugation or filtration. The presence of 80,000 to 100,000 pus cells per c. mm. increases the albumin content by 0.1 gram per 100 cc. of urine.
5. If found in urine from a female, contamination from the genital tract should be ruled out by examining a catheterized specimen.
6. In males the collection of the first half of the urine in one glass and the remainder in another will differentiate pus from the urethra from pus coming from the bladder or pelvis of the kidney.
   a. If the urine in the first glass alone is turbid, it indicates urethral pus.
   b. If the urine in both glasses is turbid, the pus is from the bladder or kidney.
   c. The presence of pus cells attached to microscopic shreds of mucus suggests a chronic prostatitis and posterior urethritis, which may, but need not, be gonorrheal.
7. It is impossible to differentiate between cystitis and pyelitis by examination of urine from the bladder.
8. A renal origin can be concluded only when the pus cells are attached to casts.
9. *Counting Leukocytes in Urine.*
   a. Shake the urine well and with a blood counting pipette (leukocyte) place a drop on the blood counting chamber.
   b. Count the leukocytes in the four large corner squares as though making a blood (leukocyte) count.
   c. Divide the sum by 4 and multiply by 10 to obtain the number in a c. mm.
   d. If there are too many to count, the urine may be diluted in the same manner as blood when making a leukocyte count.

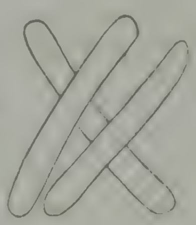
C. *Erythrocytes.*



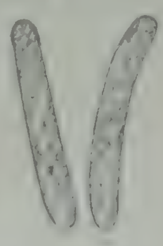
1. They vary greatly in appearance according to the physical and chemical properties of the urine and the age of the specimen.
   a. In concentrated urine they are likely to be crenated.
   b. In dilute urine they are swollen and appear as faint colorless rings.
   c. In alkaline urine they may be very small or entirely destroyed, forming masses of brownish granules.
2. If in doubt whether erythrocytes are present, add a drop of 10% acetic acid which dissolves the erythrocytes, or do a benzidine test on the sediment left in the centrifuge tube.
3. An occasional erythrocyte per low power field may be observed in normal urine. However, in the female the uterus and vagina must be eliminated as a source (examine a catheterized specimen).
4. Urine containing large numbers of erythrocytes also contains blood serum which will give a positive albumin test after removal of the erythrocytes by centrifugation or filtration.
5. Hematuria is found in severe hyperemia of the kidneys, in acute and chronic nephritis, renal tuberculosis, malignant disease of the urinary tract, hemorrhagic diseases, renal calculus, and schistosomiasis.

D. *Casts from the Renal Tubules.*

1. Casts are formed by coagulation of albuminous material in the lumen of the tubules of the kidneys. They appear as a hyaline or waxy substance, occurring in cylinders with rounded or broken ends, varying a great deal in length and thickness, but the diameter is usually uniform in each cast throughout the length. They often contain renal cells, erythrocytes, or leukocytes. They dissolve in alkaline urine.
2. Casts can be seen only with a well-darkened microscopic field.
3. *Kinds of Casts.*
   a. *Hyaline casts* are colorless, homogeneous, semitransparent, cylindrical structures with parallel sides and usually rounded ends. They indicate the mildest renal damage, a few being found after exertion and after palpation of the kidney.



   b. *Granular casts* are hyaline casts containing granules which come chiefly from the disintegration of epithelial cells of the tubules.
      1) *Finely granular casts* contain many fine granules and are grayish or pale yellow. They are the least significant of the granular type and may be found with hyaline casts when the epithelium is only slightly and perhaps not seriously affected.

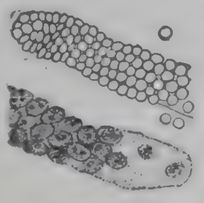


2) *Coarsely granular casts* contain larger granules and are darker in color, often being dark brown owing to the presence of altered blood pigment. They are usually shorter and more irregular in outline, frequently having irregularly broken ends. When present in considerable numbers, they point to serious glomerulonephritis. The dark brown type is most common in acute nephritis. Clumps of amorphous urates in the form of casts but not having definite outlines should not be mistaken for granular casts.

c. *Waxy casts* are grayish or colorless, highly refractive, broader than hyaline casts, appear much more solid, and are usually found broken off with square ends as if they were brittle. They are found in advanced nephritis, but mostly in amyloidosis.

d. *Fibrinous casts* resemble waxy casts, but are yellow or brown.

e. *Epithelial casts* are true hyaline casts which contain many renal epithelial cells. They indicate an active acute nephritis.



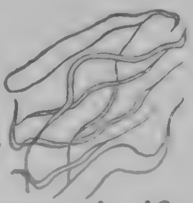
f. *Blood casts* contain large numbers of red blood cells. They indicate an active acute nephritis.

g. *Pus casts* contain pus cells. They indicate an active nephritis.

h. *Fatty casts* contain numerous globules of fat or the fat may be in very fine refractive granules. They point to a serious glomerulonephritis.

E. *Mucous Threads and Cylindroids.*

1. *Mucous threads* are long, narrow, wavy shreds from mucous surfaces. Normal in small numbers, greatly increased with irritation of any kind.



2. *Cylindroids* resemble hyaline casts, but one end tapers off to a fine filament. These are easily confused with mucous threads. When definitely consisting of hyaline material they have the same significance as hyaline casts.

F. *Diatoms.*

1. They are unicellular microscopic algae from tap water.

2. They have no significance.

G. *Bacteria.*

1. Under normal conditions urine is free from bacteria in the bladder, but becomes contaminated in passing through the urethra. Bacteria if present in large numbers may give a faint test for albumin.

2. *Various nonpathogenic bacteria,* notably Micrococcus ureae, are present in old or decomposing urine. They are easily seen with the low power objective of the microscope, but are not important. They produce a cloudiness which will not clear upon filtration through paper.

3. *Tubercle Bacilli in Urine.*
   a. See the Section on Bacteriology (p. 178) when cultures and animal inoculations are to be made.
   b. Obtain a sterile specimen (first voided in morning) in case cultures or animal inoculations are wanted later. (Do not restrict fluids.)
   c. When the specimen is more than 100 cc., proceed as follows:
      1) Centrifuge in 2 or 4 sterile 50 cc. centrifuge tubes at a high rate of speed for 45 minutes.
      2) Remove the upper 1 cc. layer with a sterile pipette, decant the remainder of the supernatant fluid, and add the urine in the pipette to the sediment.
      3) Add more urine to the same tubes and centrifuge again, repeating this process until the entire specimen is centrifuged.
      4) Concentrate by washing the sediment in the tubes into one and centrifuging.
   d. When the specimen is less than 100 cc. and concentrated, dilute with sterile distilled water until pale straw in color and then centrifuge as above.
   e. When cultures or animal inoculations are *not* to be made, dilute 2 parts of urine with 1 part of 95% alcohol instead of distilled water and centrifuge at a high rate of speed for 45 minutes. Decant supernatant fluid.
   f. Make smears of the sediment and dry in the incubator for 3 hours or overnight at room temperature; flame before staining.
   g. Add carbol-fuchsin solution and steam for 5 minutes.

   h. Let stain cool on slide and then wash off and decolorize with acid alcohol (5% nitric acid in 95% alcohol) for 5 minutes or until only a faint pink remains.
   i. Wash in water and counterstain with Loeffler's methylene blue solution for a few seconds.
   j. Rinse in water, dry, and examine.

4. *Gonococci in Urine.*
   a. Occasionally found in pus cells in the sediment when stained by Gram's method.
   b. More commonly found in stained smears of "gonorrheal threads" or "floaters."
5. *Yeast* may be found in acid urine, especially in urine containing sugar.
6. *Urine Cultures*—see Section on Bacteriology (p. 178).

H. *Parasites.*

1. The most common parasite is *Trichomonas vaginalis* from the vagina or *Trichomonas hominis* from the rectum. See Fig. 17, page 143.
   a. They are pear-shaped cells with an undulating membrane and are very motile.
   b. They range in length from 10 to 25 microns and in breadth from 5 to 15 microns.
   c. On the blunt anterior end there is a cluster of three or four flagella of equal length and on the posterior end one flagellum.
   d. *T. hominis* has a sharply pointed caudal process which is not as long as the posterior flagellum.
2. *Schistosoma haematobium.*
   a. The ova are found in the urine which must be examined immediately after being voided because they develop rapidly into larvae.
   b. The ova are more numerous in the bloody portion of the urine collected separately at the end of urination.
   c. They are 120 to 190 microns long and 50 to 73 microns broad, yellowish in color, and slightly transparent. They have a thorn-like spine at one end. See Fig. 16, page 138.
3. Hooklets and scolices of *Echinococcus granulosus* are occasionally found. See Fig. 15, page 132.

Renal Function Tests

I. Phenolsulfonephthalein Test.

A. *Principle:* This is a test primarily for tubular function; one third of the dye is filtered through the glomerulus and two thirds is excreted by the tubules.

B. *General Considerations.*

1. In normal individuals the quantity of dye excreted is independent of urine volume, but with severe renal impairment the output of dye varies directly with the urine volume and can be increased by the liberal use of water.
2. Test should not be given when cyanosis is present or after magnesium sulfate or sodium bicarbonate has been taken.
3. Acidosis decreases the excretion of dye.
4. Alkalosis increases the excretion of dye; also hyperthyroidism, because it increases blood flow through the kidneys.
5. A total excretion of more than 80 per cent suggests impaired liver function since normally the liver excretes part of the dye into the bile.

C. *Procedure.*

1. Patient may have a light breakfast and 1 glass of water to drink, but no coffee or tea.
2. Inject exactly 1 cc. of sterile phenolsulfonephthalein solution (6 mg.) intravenously and then give the patient 1 glass of water to drink.
3. Collect the urine at the end of 15 minutes and give the patient another glass of water.
4. Collect urine specimens at 1 hour and 2 hours after the injection of dye.
5. It should be ascertained that the patient has not voided for at least 1 hour before the injection of the dye.
6. The dye may be given intramuscularly and the urine collected 1 hour and 10 minutes, and 2 hours and 10 minutes after the injection. (One glass of water should be given at the time of the injection and another after the collection of the first urine specimen.)

D. *Estimation of Output.*

1. Measure quantity of specimen in a liter graduate; it must be 40 cc. or over to give dependable results. Record volume of each specimen in report.
2. Dilute to 200 cc. with tap water, rinsing out the vessel containing the urine.
3. Render alkaline by adding sufficient 10% sodium hydroxide to bring out maximal purple-red color (about 10 cc.) and then dilute with tap water to 1 liter or less if the color is slight.
4. Add 1 or 2 more drops of sodium hydroxide which will show whether the maximal color has been produced.
5. Mix well by placing hand over the top of the graduate and turning it upside down 3 or 4 times.
6. Wash and fill the open ampule of the Dunning colorimeter with the diluted urine. Filter the urine if it is cloudy.
7. Compare the color with the test ampules.
8. The per cent of dye excreted is the num-

ber on the ampule that matches the color of the diluted urine. If the dilution was less than 1 liter, divide the per cent on the ampule according to the dilution. That is, if 500 cc. divide by 2, if 250 cc. divide by 4.

9. After the sodium hydroxide is added, the estimation of dye must be made at once because an excess of alkali causes rapid destruction of the dye.
10. If bile is present, add to the urine in a graduate an equal amount of a saturated solution of barium hydroxide plus an extra 50 cc., then dilute to volume, shake, and filter.
11. If a Dunning colorimeter is not available, a Duboscq colorimeter or photoelectric colorimeter may be used.
12. *Duboscq Colorimeter.*
    a. Make a standard by placing exactly 0.5 cc. of the dye solution in a liter volumetric flask, add 10 cc. of 10% sodium hydroxide, and dilute to volume with distilled water (3 mg. of dye in 1 liter).
    b. The alkalinized urine should be diluted so that the color approximates that of the standard.
    c. Filter the diluted urine and compare with the standard set at 20.
    d. Calculate the per cent of dye excreted with the following formula and correct for dilution if the urine was not diluted to 1 liter.

$$\frac{\mathrm{RU}}{\mathrm{RS}} = \frac{50}{x} \qquad \text{or} \qquad x = \frac{50\,\mathrm{RS}}{\mathrm{RU}}$$

13. *Photoelectric Colorimeter.*
    a. The per cent of dye excretion can be obtained by reading the diluted urine (filtered) after setting the galvanometer at 100 with a blank of tap water and using filter No. 540.
    b. *Calibration of standard curve.*
        1) Make a stock dye solution by placing exactly 1 cc. of dye solution in a 100 cc. volumetric flask, add 10 cc. of 10% NaOH, and make up to volume with distilled water.
        2) Make the following dilute solutions from the stock solution, they represent the per cent of dye excreted in the urine.

Stock solution	Distilled water to	Represents per cent dye excreted
6 cc.	100 cc.	60
5 cc.	100 cc.	50
4 cc.	100 cc.	40
3 cc.	100 cc.	30
2 cc.	100 cc.	20
1 cc.	100 cc.	10

        3) Obtain the galvanometer reading for each dilution after setting the galvanometer at 100 using distilled water as a blank.
        4) Repeat several times using the same dilute solutions and then repeat using several new stock dye solutions.
        5) Average the galvanometer readings for each dilution and plot on semilogarithmic graph paper.
        6) Make a table of values according to the per cent of dye excreted.

E. *Normal Values.*

1. *After Intravenous Injection.*
   Appearance in urine: 3-5 minutes.
   After 15 minutes: 25 to 30%.
   After 1 hour: 45 to 60%.
   After 2 hours: 60 to 75%.
2. *After Intramuscular Injection.*
   Appearance in urine: 5-11 minutes.
   After 70 minutes: 40 to 60%.
   After 2 hours and 10 minutes: 60 to 75%.

F. *Pathological Findings.*

1. In chronic nephritis the retention of dye parallels the impairment of renal function and nitrogen retention in the blood.
    a. The first sign of impairment is a decrease in the amount of dye excreted at 15 minutes, even though the total excretion is normal.
    b. Elimination of 30 to 45% at the end of the first hour shows slight impairment; 10 to 30% moderate impairment; and 0-10% severe impairment of renal function.
    c. Nitrogen retention in the blood begins when the dye excretion is less than 40% in 2 hours, and the excretion of dye practically stops when the nonprotein nitrogen reaches 100 mg. per cent.
2. The test may be misleading in acute nephritis as a normal or even increased excretion may be obtained, especially in acute glomerulonephritis, or an extremely low excretion, reduced almost to zero, may be followed by complete recovery.
3. In malignant hypertension the rate is diminished as well as the total output in 2 hours.
4. In benign hypertension, hyperthyroidism, and angioneurotic edema the amount of dye excreted in 15 minutes is greater than normal, while the amount of dye excreted in 2 hours may be normal or slightly more than normal.
5. In chronic passive congestion the excretion of dye is decreased without an increase in blood nitrogen. Also low values are found in prostatic hypertrophy with hydronephrosis and in cystitis with retention of urine.

II Concentration and Water Excretion Test.

A. ***Principle:*** The kidney supplies one mechanism for regulating water balance. When there is a large intake of fluid, the kidneys excrete a large volume of very dilute urine, and thus rid the body of the excess water. When the fluid intake is small, the tubules absorb more water from the glomerular filtrate and the kidneys excrete a small amount of concentrated urine with a high specific gravity. After a sufficient number of glomeruli have been destroyed to reduce the total functioning of the kidney tissue below the factor of safety, each remaining active glomerulus produces more filtrate because of a compensatory hypertension. This filtrate passes through the tubules more rapidly than normal and thus less fluid is reabsorbed so it becomes impossible for the kidneys to excrete a urine of high specific gravity. As it is necessary for all the residual functioning glomeruli to work at full capacity for 24 hours, the day and night specimens are nearly equal in volume and have at first a fixed specific gravity around 1.017. As the disease progresses the fixed specific gravity gradually becomes lower until it reaches 1.007, the specific gravity of protein-free blood plasma.

B. ***Instructions to Patient.***

1. No extra fluids with evening meal (only one glass of water). This meal should have a high protein content.
2. No food or fluids after this time until arrival at the laboratory for the test.
3. Save first urine specimen after rising and bring it to the laboratory.

C. ***At the Laboratory.***

1. Measure volume and take the specific gravity of the first morning specimen.
2. If the specific gravity is 1.022 or over, proceed as follows:
   a. Have patient void and discard specimen.
   b. Give patient 1500 cc. of water to drink during the next 30 minutes.
   c. Collect specimens of urine 1, 2, 3, and 4 hours from the time the patient started to drink the water.
   d. Measure the volume and take the specific gravity of each specimen.
3. If the specific gravity of the first morning specimen is less than 1.022, proceed as follows:
   a. Have patient void and take specific gravity, if 1.022 or over proceed as directed under 2.
   b. If below 1.022, collect another specimen after one hour and take the specific gravity.
   c. Then give 1500 cc. of water to drink and collect hourly specimens for 4 hours.
   d. Measure and take specific gravity of each specimen.

D. ***Normal Values.***

1. First morning specimen should be concentrated to the specific gravity of 1.022 or over.
2. First hour specimen after water should have a specific gravity of 1.001 to 1.003 and a volume of about 400 cc.
3. Each hour thereafter the volume is less with increasing specific gravity.
4. At the fourth hour the specific gravity should be between 1.012 and 1.016 with a volume of about 100 cc.
5. The total volume of the urine should be 80 to 120% of the intake of water (about 1200 cc.).

E. ***Pathological Findings.***

1. The first evidence of kidney damage is an inability of the kidney to excrete urine of a specific gravity of 1.022 or higher.
2. Later the kidney is not able to excrete a urine of low specific gravity (1.003) after drinking water.
3. As the disease progresses the specific gravity of the first specimen after rising approximates that of the first specimen after drinking water.
4. The specific gravity of the concentrated specimen reaches 1.020 before the phenolsulfonephthalein test is decreased; and as low as 1.010 before the blood nonprotein nitrogen is elevated.

III. Mosenthal's Concentration Test.

A. ***Principle:*** Normally the urine varies greatly in its volume and specific gravity at different periods of the day. This shows the ready response of the kidney to the varying demands for elimination of water and solids. The diseased kidney loses this adaptive power to a greater or lesser degree and the urine excreted consequently remains of an almost uniform concentration from hour to hour.

B. ***Method.***

1. Patient eats 3 regular meals during the day and drinks the usual amount of fluids.
2. Patient voids at 8 A.M. and the urine is discarded.
3. Urine specimens are collected at the following hours: 10 A.M., Noon, 2 P.M., 4 P.M., 6 P.M., and 8 P.M.
4. Measure the volume and take the specific

gravity of each specimen.

5. All urine passed from 8 P.M. until 8 A.M. (12 hours) is placed in one container, measured, and the specific gravity taken.

C. *Normal Values.*

1. Night specimen should not exceed 575 cc. in volume and should not be more than 75 per cent of the day volume. The specific gravity should not be lower than 1.018.
2. In the day specimens the specific gravity should vary by 9 points or more, reaching 1.020 in one or two specimens.

D. *Abnormal Findings.*

1. The first evidence of impaired kidney function is nocturnal polyuria; that is, the night urine exceeds 575 cc. and its specific gravity is lower than 1.018.
2. The next evidence of impairment is a fall in specific gravity below 1.018 in any of the day specimens.
3. The level at which the specific gravity is fixed becomes lower as the functional impairment increases.
4. In chronic passive congestion there is a fixed high specific gravity and oliguria.

IV. Blood Urea Nitrogen to Nonprotein Nitrogen Ratio.

A. *Method.*

1. See method for blood urea nitrogen (B U N), page 278, and nonprotein nitrogen (N P N), page 272.
2. Calculation:

$$\frac{\text{Urea N} \times 100}{\text{NPN}}$$

B. *Interpretation.*

1. The normal ratio is 40 to 47 with extremes at 35 and 50.
2. In a slight or moderate degree of renal insufficiency, the selective concentration of urea by the kidney is impaired, resulting in a faster increase in blood urea than in nonprotein nitrogen, thus giving a higher ratio.
3. In maximal impairment of renal function there is a ratio of 80 or more.
4. The ratio is decreased in hepatic insufficiency, eclampsia, and toxemias associated with a rapid breakdown of body protein.
5. Normal pregnancy produces a ratio slightly lower than normal (average 33.1).

V. Urea Clearance Test.

A. *Method.*

See page 281 in Section on Chemistry for method and calculation.

B. ***Principle:*** The rate of excretion of urea in urine per minute is directly proportional to the blood urea content. It is reported in per cent of an established normal for a body surface area of 1.73 sq. meters.

C. *Interpretation.*

1. Normal is 60 per cent or more.
2. Mild impairment between 40 and 60 per cent.
3. Moderate impairment—20 to 40 per cent.
4. Severe impairment—below 20 per cent.

Correlation of Clinical and Laboratory Findings with the Pathology of the Kidneys

The kidneys perform at least three important functions: (1) they are the chief means of excretion of nitrogenous waste products from protein metabolism; they are essential factors in the maintenance (2) of the water balance, and (3) of the acid-base balance in the body.

The walls of the glomeruli are permeable to all the crystalloidal substances in the blood plasma, including urea, uric acid, creatinine, sodium chloride, sugar, etc. These substances are eliminated by glomerular filtration. The excretion of water by the normal kidneys, also by glomerular filtration, is directly proportional to the intake of water. They are also the chief channels of elimination of water in the reduction of edema.

The role of the kidneys in the maintenance of the acid-base balance of the body is accomplished by three processes. (a) They transform a part of the sodium alkaline phosphate which is filtered out of the blood by the glomeruli into sodium acid phosphate and return to the body one atom of sodium (fixed base) for each molecule thus transformed, according to the following formula:

$$Na_2HPO_4 + H_2CO_3 = NaH_2PO_4 + NaHCO_3$$

The sodium acid phosphate is excreted in the urine; the bicarbonate is returned to the blood. This is further shown by the relative concentrations of the acid and alkaline phosphates in the plasma and urine, as follows:

In plasma $\frac{NaH_2PO_4}{Na_2HPO_4}\ \frac{1}{4}$; In urine $\frac{NaH_2PO_4}{Na_2HPO_4}\ \frac{9}{1}$

(b) When there is a threatened dangerous depletion of fixed base (chiefly sodium) in the body, the kidneys are capable of performing an emergency function by transforming urea into ammonia and thus supplying a volatile base. (c) In the acidosis of diabetes the diacetic and beta-oxybutyric acids in the plasma are combined with fixed base (sodium). The kidneys are capable of dissociating these salts and eliminating up to 11 per cent of the diacetic and up to 5 per cent of the beta-oxybutyric acids as free acid and of returning to the blood the dissociated

odium to maintain the alkali reserve. Inter-
rence with these three important processes to
aintain acid-base balance accounts for the oc-
urrence of acidosis in chronic nephritis.

The formation of urine begins in the glomeruli
nd is completed in the distal convoluted tubules.
nder the hydrostatic pressure of the blood in
ne glomerular capillaries an ultrafiltrate is pro-
uced consisting of a watery solution of all the
rystalloidal substances in the plasma in the same
oncentration in which they are present in the
lasma. As this filtrate passes through the tubules,
ll the sugar, about 98.5 per cent of the water,
nd varying amounts of sodium chloride, urea,
nd other substances are absorbed by the tubular
pithelium. The final product, urine, is a greatly
oncentrated solution that is hypertonic to the
lood.

In order to perform their function efficiently
he glomeruli must receive a sufficient amount of
lood under adequate filtration pressure to pro-
uce an ultrafiltrate and the tubules must re-
eive a blood supply sufficient to maintain their
utrition. In spite of differences in their charac-
eristic pathology, all the kidney diseases which
ead to renal insufficiency and uremia have in
ommon interference with the blood supply to
ne glomeruli and tubules. Since the tubules are
upplied with blood which has first passed
rough the glomeruli, any condition which re-
uces the blood supply to the glomeruli will
so interfere with the nutrition of the tubules,
ich as glomerulonephritis, chronic pyelonephri-
s, and the changes in the afferent arteries to
ie glomeruli that accompany hypertension.

Glomerulonephritis is of two types: acute and chronic. Subacute glomerulonephritis is a somewhat indefinite pathologic condition. It will not be considered here because it resembles in many of its features the corresponding chronic disease from which it may be difficult to differentiate clinically.

. ***Acute glomerulonephritis*** occurs in two forms so far as the pathology of the disease is concerned: exudative and proliferative.

Acute exudative glomerulonephritis may occur as a sequel of scarlet fever as well as in other conditions. It is characterized by hyperemia of the glomeruli whose capillaries are rich in polymorphonuclear leukocytes. Similar cells are seen in the capsular space and in the tubules. The stroma of the kidneys is usually more or less edematous. This type of the disease is only rarely fatal and does not often become chronic. Perhaps the reason for this is that the exudate is drained away down the tubules and there is little stimulus to induce fibrosis and destruction of the glomeruli. The laboratory and most important clinical manifestations of this disease are reasonably characteristic.

a. The urine is usually somewhat reduced in amount and its specific gravity is increased. The glomeruli are damaged and albumin, red blood cells sometimes in sufficient numbers to give it a reddish or smoky color, pus cells, and casts are present in the urine.

b. Edema, usually moderate and affecting chiefly the eyelids and face, is relatively common. This edema is apparently due to widespread damage to capillaries through whose hyperpermeable walls water and serum albumin escape. The latter holds the water in the tissues by upsetting the balance between the osmotic pressure of the plasma proteins and that of the tissue fluids.

c. A moderate degree of hypertension is not infrequent. This is apparently due to ischemia of the renal tissue resulting from interference with the circulation through these organs by increase of intrarenal pressure caused by edema of the renal stroma and cloudy swelling of the tubular epithelium. This is also the cause of the oliguria, anuria, and renal insufficiency which occur in severe and fatal cases.

2. *Acute proliferative glomerulonephritis* sometimes follows infections of the tonsils and other structures in this region. Frequently there is no discoverable etiology. This type of the disease is fatal or passes into the chronic type more frequently than does the exudative form of acute glomerulonephritis. It is characterized by proliferation of the endothelial lining of the glomerular capillaries and the connective tissue cells in their walls. The glomeruli are, therefore, more cellular than normal and contain very little blood. This change causes marked interference with the flow through both the glomerular and peritubular capillaries. The laboratory and clinical findings are not as distinctive or characteristic as in the exudative type.

   a. The urine contains less albumin and fewer red blood cells, leukocytes, and casts than in the exudative form. The total quantity excreted per day is reduced.

   b. Edema is not so likely to occur as in the exudative type of the disease.

   c. Moderate hypertension is usual and is due, probably, to ischemia of the renal substance resulting from the obstruction of the flow of blood through the glomeruli.

d. Increase in the urea and nonprotein nitrogenous substances of the blood is common and is due to diminished filtration through the occluded glomeruli through which alone these substances can be excreted.

B. *Chronic glomerulonephritis* may be accompanied by marked ("nephrotic") edema or by progressive retention of nitrogenous waste products (the azotemic type).

1. *In chronic glomerulonephritis with edema*, there is little interference with the flow of blood through the glomeruli in the earlier stages of the disease. As a result of some cause not now known and of some change in the glomerular walls that cannot be demonstrated with certainty, the walls of the glomeruli become hyperpermeable and permit not only the usual ultrafiltrate to pass through but also serum albumin to which the normal glomeruli are impermeable. The laboratory and clinical findings are characteristic.
   a. The urine contains an abundance of albumin, its specific gravity is usually somewhat increased, and it contains many casts but relatively few red blood cells and leukocytes.
   b. Because of the constant loss of large amounts of serum albumin in the urine the amount of this protein in the plasma is reduced, often to a level below that of the globulin ("inversion of the albumin-globulin ratio").
   c. As a result of the marked reduction of the plasma proteins their osmotic pressure in the blood is reduced below a critical level at which more fluid leaves the systemic capillaries than returns to them. Hence very extensive edema of the subcutaneous tissue may occur, often with accumulation of large amounts of fluid in the serous cavities.
   d. During this stage of the disease there is no hypertension because there is no ischemia of the kidneys and no increase in nonprotein and urea nitrogen of the blood because there is little interference with filtration through the glomeruli through which these products are excreted.
   e. If the patient survives for a sufficient time, the pathologic and clinical picture may change completely and come to resemble, in all essential respects, that of the azotemic type now to be described.
2. *The azotemic type* of chronic glomerulonephritis may follow the proliferative form of acute glomerulonephritis, but more frequently develops insidiously without any acute manifestations. The primary change occurs in the glomeruli as a proliferation of their constituents—endothelium, connective tissue, and perhaps epithelium. The ultimate result is the progressive transformation of the glomeruli into bloodless scars. This not only prevents filtration through the affected glomeruli but also shuts off the blood supply to the convoluted tubules. As these changes progress the laboratory and clinical findings vary with the stage of the disease.
   a. The amount of albumin in the urine is relatively small in proportion to that present in the type of glomerulonephritis with edema. Casts are abundant and of many varieties. The specific gravity of the urine may be normal at first but gradually is lowered, becoming "fixed" at a level between 1.017 and 1.007, that is, the kidneys become unable to secrete either a very dilute or a very concentrated urine, no matter what the intake of water.
   b. The changes in the glomeruli reduce the blood supply to the tubules, i.e., cause ischemia of the kidneys with resulting progressively increasing hypertension. The high blood pressure, in time, leads inevitably to hypertrophy of the heart and the patient may die from congestive heart failure with extensive cardiac edema.
   c. The total number of functioning glomeruli is reduced by their progressive destruction. When this destruction exceeds the factor of safety or functional reserve of the kidneys, renal failure becomes manifest with increase of nonprotein nitrogen, urea, and creatinine of the blood to very high levels and the patient dies of uremia.

II. In pyelonephritis renal tissue is destroyed and changes occur both in the blood vessels and in the parenchyma and stroma not only in the portions actually the seat of inflammation and abscess formation but also in a zone of varying width about each inflamed area. The destroyed renal tissue is replaced by scar tissue and the total amount of parenchyma capable of functioning is decreased. The alterations in the arteries and arterioles reduce the blood supply of the intact renal tissue, i.e., produce ischemia. This is followed by hypertension which may produce progressive changes similar to those in essential hypertension and with the same general effects on renal function.

TABLE 4. LABORATORY FINDINGS IN KIDNEY DISEASE

	Urine Findings	Blood Chemistry	Renal Function	Systemic Changes	Prognosis
Acute Glomerulonephritis.	Oliguria. Acid. Sp. gr. high. Dark red and cloudy. Albuminuria (1-1.5 gm.%). Casts: hyaline, granular, brown granular, blood, pus, and epithelial. Blood and pus.	Nitrogen retention in proportion to severity. Urea to 120 mg.%. Uric acid 3-6 mg.%. Creatinine 1-7 mg.%.	Impairment in proportion to severity.	Blood pressure increased. Edema present. Eyeground changes absent. Mild anemia.	Frequently recover. May be fatal.
Chronic Glomerulonephritis without Edema (Azotemic type).	Polyuria. Acid. Sp. gr. low, later becoming fixed. Pale yellow and clear. Albuminuria (trace). Casts: hyaline and granular. Blood present at times. Late in disease: oliguria.	Nitrogen retention. First stage: intermittent. Second stage: moderate. Third stage: marked and constant.	Impaired. First stage: slight. Second stage: moderate. Third stage: severe to absolute insufficiency.	Blood pressure increased. Edema absent unless cardiac failure is present. Eyeground changes absent or present. Moderate to severe anemia. Heart enlarged. Late: chronic uremia.	Repeated exacerbations may occur. Fatal in 2 to 25 years.
Nephrosis and Chronic Glomerulonephritis with Edema (Nephrotic type).	Oliguria in proportion to the edema. Sl. acid or alk. Sp. gr. normal to very high. Normal appearance. Marked albuminuria (1-6 gm.%). Casts: hyaline and granular. Occasional blood cell. (In lipoid nephrosis there are no casts, the cells contain lipoid, and there are free lipoid granules.)	Nitrogen not increased. Chlorides increased. Cholesterol increased (0.3 to 2 gm.%). Total protein decreased (⅛ to ½). Albumin-globulin ratio inverted because so much albumin is lost in the urine.	Normal unless edema interferes with tests.	Edema marked. No increase in blood pressure. No eyeground changes. B.M.R. low (—20%). Sedimentation rate of R.B.C. greatly increased. Faulty protein metabolism. No inflammation of glomeruli. More marked in young individuals.	May recover. Conditions may last from months to many years or develop into the azotemic type.
Arteriolosclerotic without Insufficiency (Benign hypertension).	Early stage: normal. Late stage: trace of albumin with a few hyaline and granular casts.	Normal unless cardiac failure is present.	Normal unless cardiac failure is present.	Blood pressure increased. Edema absent unless cardiac failure is present. Eyeground changes absent. Cardiac dilatation and failure are rare, while hypertrophy is common.	Good, unless complicated by cardiac failure.
Arteriolosclerotic with Insufficiency (Malignant hypertension).	Early stage: normal. Late stage: like chronic nephritis without edema.	Like chronic nephritis without edema.	Like chronic nephritis without edema.	Blood pressure high. Edema absent unless cardiac failure is present. Eyeground changes marked. Cardiac hypertrophy, dilatation, and failure are common. Apoplexy common. Chronic uremia late. Anemia marked.	Poor, but condition may last from a few years to a decade. Fatal within 6 months—after absolute insufficiency occurs.

III. Nephrosclerosis; Hypertension. The pathological equivalent of long continued clinical hypertension is sclerosis of the arterioles. While, in hypertension, arterioles may be involved in widely separated parts of the body, it is only in the kidneys that this change is constantly associated with high blood pressure. In these organs the afferent arterioles to the glomeruli become thickened and their lumens narrowed. As long as the systemic blood pressure is high enough to supply the glomeruli with a sufficient amount of blood under adequate hydrostatic pressure to maintain glomerular filtration at normal levels there will be no disturbance of renal function. This is "benign hypertension." The systemic blood pressure compensates for the changes in the arterioles. When, as a result of marked lowering of systemic blood pressure, as in congestive heart failure, or of extreme changes in the afferent arteries so great and so extensive that an adequate blood supply to the glomeruli cannot be maintained, renal insufficiency occurs and the patient may die of uremia. This is "malignant hypertension." But even in the benign type of this condition there is slow progressive reduction in the amount of functioning renal tissue.

A. ***In benign hypertension*** the high blood pressure compensates for the narrowing of the lumens of the afferent arteries and there is little evidence of reduction of renal function. There may be moderate polyuria. The urine may contain a trace of albumin and a few hyaline casts and its specific gravity may be moderately low. The nonprotein nitrogen of the blood is usually within normal limits.

B. Clinically, ***malignant hypertension*** closely resembles the late stage of the azotemic type of chronic glomerulonephritis from which it can be differentiated with difficulty. Prior to the development of congestive heart failure, which may occur in both conditions, the blood pressure is usually higher in malignant hypertension than in chronic glomerulonephritis. Otherwise, there is, in both conditions, polyuria with low and fixed specific gravity, slight albuminuria, the presence of casts of various types, and marked increase in the nonprotein nitrogen of the blood. The similarity of the effects of these two conditions upon the function of the kidneys is the result of markedly reduced glomerular filtration. In chronic glomerulonephritis this is due to primary destruction of the glomeruli themselves; in malignant hypertension, to inadequate glomerular circulation and to destruction of glomeruli secondary to the changes in the afferent arteries.

Hematology

The blood consists of plasma, in which are suspended red corpuscles, white corpuscles, and blood platelets. All of these may be affected by changes either in the blood forming organs or in other tissues of the body. Therefore, a blood examination is very important in all diseases.

Although blood examinations, next to urine examinations, are the most frequent tests performed in the laboratory, they are also the ones in which there are the greatest inaccuracies. If the tests are to be used for clinical interpretation, it is of the greatest importance to use clean instruments and to develop an accurate technique.

Table 5. Normal Hematological Values in Adults

Erythrocytes:			
Men	4.5 - 6.0 million	Diff. Leukocyte Count:	
Women	4.0 - 5.5 million	Neutrophils	
Leukocytes	4,000 - 10,000	Band	2 - 6%
Hemoglobin (Hgb.):		Segmented	50 - 70%
Men	14 - 18 gm.%	Eosinophils	1 - 3%
Women	12 - 16 gm.%	Basophils	0 - 1%
Color Index	0.90 - 1.10	Lymphocytes	20 - 40%
Mean Corpuscular Hgb. (M.C.H.)	27 - 32 micromicrograms	Monocytes	2 - 8%
		Coagulation Time:	
Cell Pack (hematocrit):		Lee and White	5 - 10 min.
Men	40 - 54%	Other methods	2 - 6 min.
Women	37 - 47%	Bleeding Time (Ivy's method)	2 - 125 sec.
Volume Index	0.90 - 1.10		
Mean Corpuscular Volume (M.C.V.)	82 - 92 c. microns	Capillary Resistance	10 or less petechiae in 8 min.
Saturation Index	1.0		
Mean Corpuscular Hgb. Conc. (M.C.H.C.)	32 - 36%	Clot Retraction Time	1 - 24 hrs.
		Prothrombin Time	above 70%
Mean Corpuscular Diameter (M.C.D.)	6.7 - 8 microns	Sedimentation Rate:	
		Men	0 - 9 mm.
Reticulocytes	0.5 - 1.5%	Women	0-20 mm.
Platelets	200,000 - 400,000	Fragility of Erythrocytes	0.46 to 0.30% NaCl

Collection of Blood

I. Skin Puncture (Capillary Blood).

A. *Method.*

1. In adults use the patient's finger or the lobe of the ear, choosing a site free from local circulatory changes (pallor, cyanosis, edema, or inflammation).
2. In infants use the great toe.
3. Rub the part well with 70 per cent alcohol or antiseptic wash and *dry*.
4. Make a good stab with a quick motion, using a sterile, dry, sharp Hagedorn needle (No. 3). When withdrawing, cut down across the lines of the skin with the sharp edge of the needle. A lancet may be used.
5. The cut should be deep enough so that the *blood flows freely without squeezing the ear or finger.*
6. The first drop should be wiped away with a dry sponge and a separate fresh drop used for each sample.

B. *Examinations Employing Capillary Blood.*

1. Blood count: erythrocyte, leukocyte, and differential.
2. Hemoglobin.
3. Bleeding and coagulation time.
4. Blood typing.
5. Reticulocyte count.
6. Platelet count.
7. Thin and thick smears for malaria.
8. Smear for basophilic aggregations (stippling of erythrocytes).

II. Venipuncture.

A. *Method.*

1. Cleanse the skin over a prominent vein at the elbow with antiseptic wash.
2. Place a tourniquet around the arm above the elbow; request the patient to vigorously open and close his hand several times.
3. The size of the syringe to be used is determined by the amount of blood desired.

4. Generally a 1¼ inch needle, 21 gauge, with a medium bevel, is used.
5. Both the needle and syringe must be sterile and dry.
6. Draw the skin tense over the vein with the thumb of the left hand. With a quick thrust puncture the skin and vein with the bevel of the needle up.
7. Do not move the needle or syringe after the needle is in the vein, but release the tourniquet.
8. Withdraw blood by slow traction on the plunger of the syringe.
9. When ready to remove syringe, apply a sterile sponge over the needle.
10. Remove needle; then have patient press sponge on puncture wound for 5 minutes.
11. Remove needle from syringe and gently empty blood in a sterile test tube to clot or in a bottle containing an anticoagulant, shaking well to prevent clotting.
12. Keep bottle stoppered to prevent evaporation.
13. Wash syringe and needle at once with cold water to remove blood before it coagulates.
14. The bottle containing the oxalated blood must be shaken for 3 minutes immediately before using the blood for a test.

B. *Anticoagulant Used in Hematology.*

1. It is very necessary to have the correct proportion of blood to anticoagulant to prevent shrinkage of the cells which would give incorrect values.

2. *Anticoagulant Solution.*

Ammonium oxalate	12 gm.
Potassium oxalate	8 gm.

Distilled water to make 1 liter.

   a. Pipette 0.3 cc. of the solution into a one-half ounce bottle and warm (not over 70°C.) until the water is completely evaporated.
   b. This amount prevents 3 cc. of blood from clotting. If more or less blood is used, it is important to use more or less anticoagulant solution in this same proportion.

C. *Examinations Employing Venous Blood.*

1. *Whole Blood (not oxalated).*
   a. Clot retraction time.
   b. Fragility test.
   c. Smear for differential count.
   d. Coagulation time (Lee and White).
2. *Whole Blood (oxalated).*
   Tests must be made within the following specified time after drawing the blood.

Erythrocyte count	24 hrs.
Leukocyte count	24 hrs.
Hemoglobin	24 hrs.
Cell pack (hematocrit)	3 hrs.
Sedimentation rate	1 hr.
Prothrombin time	30 min.
Reticulocyte count	1 hr.

Counting of Blood Cells

I. Erythrocyte Count.

A. *Collecting Blood.*

1. Use pipette marked 101 above the bulb. It must be clean and dry.
2. Make a skin puncture as described above.
3. Hold the pipette in a horizontal position and draw the blood exactly to the 0.5 mark.
4. Wipe off any blood which adheres to the outside of the pipette with a cloth without drawing blood from the pipette itself.
5. If the blood is drawn past the 0.5 mark, never more than 2 mm., the blood can be adjusted back to the mark by stroking the tip of the pipette with the finger or a piece of thin rubber. Do not use a cloth as it tends to withdraw the fluid portion of the blood leaving a higher concentration of cells.
6. Draw a small bubble of air into the capillary of the pipette.
7. Insert the tip of the pipette into the diluting fluid (Gower's or Hayem's solution, see p. 356), hold the pipette vertical, and fill to the 101 mark, sucking very slowly just before reaching the mark. Rotate pipette between thumb and finger slowly while filling to mix blood and solution.
8. When completely filled, bring pipette to a horizontal position and place finger over the tip before removing the rubber tubing.
9. Hold the pipette horizontally between the thumb and middle finger and shake with a rotatory movement until well mixed.

B. *Counting Erythrocytes.*

1. The ruled area of the counting chamber and the cover glass must be carefully cleaned and absolutely free from dust, lint, and grease.
2. Place the counting chamber on a horizontal surface and lay the cover glass in place over the ruled area.
3. Shake the pipette 3 minutes, then blow out one third of its contents and wipe off the tip so no fluid is present.
4. Place a finger over the upper end of the pipette and touch the tip to the ruled area at the edge of the cover glass. Remove the finger and allow the fluid to fill the space

by capillarity. When the fluid has flowed three fourths of the way across, withdraw the pipette as there is enough fluid present to fill the space. (No time must elapse between shaking the pipette and filling the counting chamber.)

5. If any fluid runs over into the moat or if there are any air bubbles present, the slide must be cleaned and filled again.
6. Allow 3 minutes for the corpuscles to settle.
7. First find the small center squares under low power and then count under high power.
8. Do not touch the cover glass with the lens; this can be avoided by always focusing up instead of down.
9. If the cells are not evenly distributed, clean the counting chamber, shake pipette, and fill the chamber again.
10. Count 80 small squares, 5 groups of 16 in the central ruled area, and to the sum add 4 ciphers. See Fig. 2.

    Calculation:

    Cells counted × 10 (depth) × 5 (1/5 of sq. mm. counted) × 200 (dilution) = erythrocytes per cubic millimeter.
11. In order to avoid confusion in counting cells which touch the sides of the group of 16 squares, count all cells which touch the upper and left outer double line of the group as if they were inside the square and neglect those cells which touch the lower and right inner line. See Fig. 3.

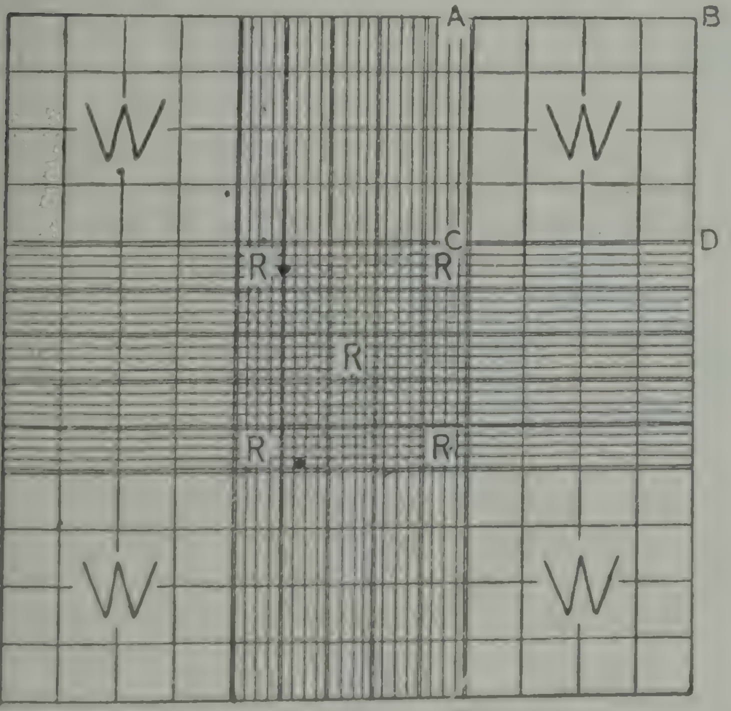


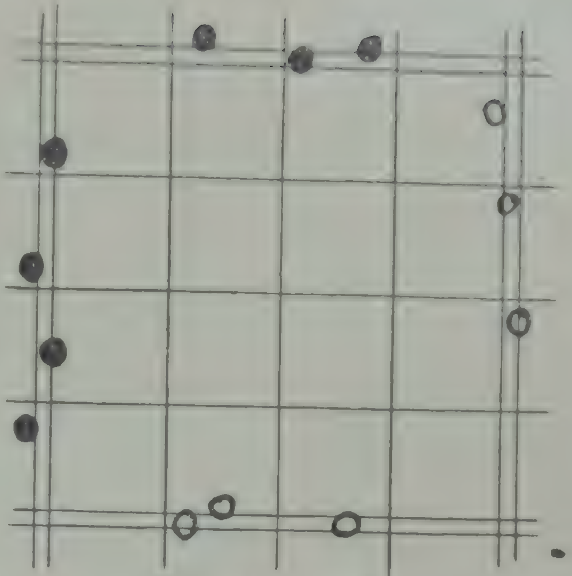
Fig. 2. Improved Neubauer Ruling on a Hemocytometer.

W = Areas to be counted under low power for leukocytes.

R = Areas to be counted under high power for erythrocytes.

Area ABCD = 1 sq. mm.

Small black square = 1/400 sq. mm.



● Cells to be counted.

○ Cells not to be counted.

Fig. 3. Method of Counting Cells Touching Double Lines on a Hemocytometer.

12. If the counting chamber has 3 lines outlining the group of 16 squares, the middle line is the boundary.
13. If the number of cells in each group of 16 small squares varies more than 16, the count must be discarded and the counting chamber filled with a fresh drop of fluid from the reshaken pipette.
14. If the first count is not within normal limits, put a fresh drop on the counting chamber and count again. Always shake the pipette well and discard 4 or 5 drops before adding a drop to the counting chamber.
15. If there is a severe anemia, the blood may be drawn to the 1 mark on the pipette and then diluted to the 101 mark. The number of corpuscles counted would then be multiplied by 5000.
16. In case of autoagglutination of the cells, use an 0.85% solution of NaCl instead of Gower's solution for making the dilution.

C. *Interpretation of Erythrocyte Counts.*

1. *Normal Values.*
    a. Men: 4,500,000—6,000,000 per c. mm. of blood.
    b. Women: 4,000,000—5,500,000 per c. mm. of blood.
    c. Normal women in late pregnancy—3,000,000 to 5,000,000 per c. mm. of blood.
    d. Children—see Table 6, page 40.
2. *Abnormal Values:*
    a. Increased (polycythemia) in dehydration, erythremia (polycythemia vera), certain chronic heart diseases, cases of acute poisoning, pulmonary fibrosis, and Ayerza's disease.
    b. Decreased (oligocythemia) in anemias, leukemia, and after hemorrhage when the blood volume has been restored.
    c. See blood findings in disease, page 93.

II. Leukocyte Count.

A. *Collecting Blood.*

1. Use a clean, dry pipette with 11 marked above the bulb and follow the technique described under erythrocyte count.
2. A larger drop of blood is required, because the bore of the leukocyte pipette is much larger than that of the erythrocyte pipette.
3. Draw blood exactly to the 0.5 mark and wipe off the tip as described under erythrocyte count.
4. Place in the diluting fluid (1% HCl), hold the pipette vertical, and fill slowly to the mark 11.
5. Shake pipette until well mixed.

B. *Counting Leukocytes.*

1. Follow directions as given in 1 through 5 under counting erythrocytes, except that only 2 or 3 drops are discarded from the pipette.
2. The pipette is held practically horizontal while filling the counting chamber to keep too much fluid from running under the cover glass.
3. Allow 1 minute for the leukocytes to settle.
4. Count the 4 corner groups of 16 squares under low power. See Fig. 2. This makes 4 sq. mm. counted, so that the sum of the number of cells counted is multiplied by 50 for the total leukocytes.

   Calculation:

$$\frac{\text{Cells counted} \times 20 \text{ (dilution)} \times 10 \text{ (depth)}}{\text{Number of sq. millimeters counted (4)}} = \text{leukocytes per cubic millimeter.}$$

5. If the number of cells for each of the 4 groups of 16 small squares varies more than 12, the count must be discarded and the counting chamber filled with a fresh drop of fluid.
6. If the first count is not within normal limits, put a fresh drop on the counting chamber and count again. Always shake the pipette well and discard a drop before filling the counting chamber.
7. If the count is from 50,000 to 500,000, collect the blood in an erythrocyte pipette, drawing the blood to the 1 mark. Count like an ordinary leukocyte count but multiply by 250. With counts greater than 500,000, draw the blood to the 0.5 mark and multiply the number of cells counted by 500.
8. If the count is less than 3,000, make a 1 to 10 dilution in the leukocyte pipette and multiply by 25.
9. When a large number of nucleated erythrocytes are present, a correction of the leukocyte count must be made from the differential count as follows:

$$a = \frac{b \times c}{c + d}$$

a = corrected leukocyte count.
b = uncorrected leukocyte count.
c = 100 (leukocytes counted in differential count).
d = number of nucleated erythrocytes per 100 leukocytes in the differential count.

C. *Interpretation of Leukocyte Counts.*

1. *Normal Values:*
   a. Adults—capillary blood, 5,000 to 10,000 per c. mm. of blood. Venous blood may be 1,000 cells lower than capillary blood.
   b. Normal women in late pregnancy—7,000 to 18,000 per c. mm. of blood.
   c. Children—see Table 6, page 40.
   d. The count varies with the condition of the patient and the time of day.
      1) It is lowest in the morning and gradually rises until the middle of the afternoon.
      2) Decreases with age and in resting condition.
      3) Increases after strenuous exercise and after cold baths.
2. *Abnormal Values*—see blood findings in disease, page 91.

III. Sources of Error in Erythrocyte and Leukocyte Counts.

1. Blood taken from cyanosed area.
2. Too much pressure on ear or finger while taking count.
3. Dirty or wet pipettes.
4. Volume of blood not measured carefully or air bubbles in the pipette.
5. Removing blood from tip of pipette with a cloth instead of finger or piece of rubber.
6. Too slow manipulation, allowing some of the blood to clot.
7. Inaccurate dilution: loss of cells in diluting fluid, incorrect final volume, or further dilution by saliva from tubing.
8. Diluted cells not thoroughly mixed.
9. Failure to expel several drops before filling counting chamber.
10. Incorrect filling of the counting chamber: allowing too much time to elapse between shaking the pipette and filling the chamber, too much or too little fluid, uneven distribution of the cells, or air bubbles present.
11. The presence of yeasts or dirt in the diluting fluid. Faulty diluting fluid causing hemolysis.
12. Touching cover glass with the objective.
13. Clumping of cells.

4. Inaccurate calculation of cells counted.
5. Inaccurate counting chambers and pipettes.
16. Incorrect area counted due to misinterpretation of ruled area on the counting chamber.
17. Inaccurate leukocyte count due to counting nucleated erythrocytes.

IV. Cleaning the Counting Chamber and Pipettes.

A. *Counting Chamber.*

1. Clean with water immediately after using; occasionally wash with soap and water.
2. Dry with a soft lint-free cloth.

B. *Pipettes.*

1. Remove the rubber tubing from the pipette and use a suction pump to aspirate the following fluids through the pipette in the order given:
   a. Distilled water until all traces of blood are removed.
   b. A small amount of acetone.
   c. Air until the pipette is dry.
   d. Alcohol and then ether may be substituted for acetone.
2. If properly cleaned and dried, the bead in the bulb should move freely.

Hemoglobin

I. Hemoglobin Values.

A. *Standards.*

1. Different methods use different standards as a working basis and the instruments are calibrated so that a certain number of grams equal 100 per cent.
   Sahli-Hellige—14.5 gm. per 100 cc. of blood.
   Newcomer—16.92 gm. per 100 cc. of blood.
   Dare (new)—16.0 gm. per 100 cc. of blood.
   Tallqvist—15.8 gm. per 100 cc. of blood.
2. If the standard is known, the precentage or grams of hemoglobin per 100 cc. of blood can be figured from the following formulas:

$$\text{Per cent Hgb.} = \frac{\text{grams Hgb.} \times 100}{\text{standard}}$$

$$\text{Grams Hgb.} = \frac{\%\ \text{Hgb.} \times \text{standard}}{100}$$

B. **Reports** should be made in grams of hemoglobin per 100 cc. of blood instead of per cent because of the different standards for per cent on the various instruments. The newer instruments are made to read directly in grams.

II. Acid Hematin Methods for Hemoglobin Determination.

A. *Sahli-Hellige Method.*

1. A Sahli-Hellige hemoglobinometer is used.
2. Place 5 drops of 1% hydrochloric acid in the graduated tube.
3. Obtain a large drop of blood and draw it into the Sahli pipette exactly to the 20 c. mm. mark.
4. Wipe off any blood which adheres to the outside of the pipette with a cloth without drawing blood from the pipette itself.
5. If the blood is drawn slightly beyond the mark, adjust it to the mark by stroking the tip of the pipette with the finger.
6. Blow the contents of the pipette into the hydrochloric acid in the tube and rinse the pipette well by drawing up the acid in the tube several times.
7. Let stand 4 minutes for the hemoglobin to change to acid hematin.
8. Dilute the fluid with distilled water drop by drop, mixing after each addition, until it matches the color of the standard comparison tube.
9. The final reading must be made at exactly 5 minutes after the blood is added to the acid.
10. The graduations at the bottom of the meniscus indicate the percentage and grams of hemoglobin; 100 per cent corresponds to 14.5 gm. of oxyhemoglobin in 100 cc. of blood.

B. ***Haden-Hausser Method.***

1. A Haden-Hausser hemoglobinometer is used.
2. Dilute the blood 1 to 20 in a leukocyte diluting pipette with 1% hydrochloric acid and mix. Follow directions under leukocyte count.
3. Let stand 30 minutes for the hemoglobin to change to acid hematin.
4. Clean the comparator slide and cover glass.
5. Assemble them by applying the cover glass with the beveled side down and with the lower and left edges in contact with the metal frame.
6. Discard 2 or 3 drops from the pipette if it has not been used for a leukocyte count.
7. Fill comparator slide by letting several drops run into the wedge-shaped trough which will fill by capillary action.
8. Place slide in the hemoglobinometer and match the fluid with the color standard (lower half of slide) which reads in grams per 100 cc.
9. If the hemoglobin is below 7.5 gm., fill another pipette, diluting the blood 1 to 10 and divide the reading by 2.
10. Use the daylight filter (D) for daylight and the Mazda filter (M) for electric light.
11. No correction of the reading is necessary if the pipette has stood 30 minutes or longer.

If it has stood only 10 minutes, add 4%, 15 minutes 3%, 20 minutes 2%.

C. *Newcomer's Method.*

1. A Duboscq colorimeter, Newcomer filters, and a Sahli pipette are used.
2. Place the blue filter either in the eyepiece or over the eyecap diaphragm of the colorimeter.
3. Adjust the colorimeter for even illumination using skylight.
4. Place the yellow filter under the left-hand cup.
5. Obtain a large drop of blood and draw it into a Sahli pipette exactly to the 20 c. mm. mark.
6. Wipe blood off the outside of the pipette without drawing blood from the pipette itself and blow its contents into 10 cc. of 1% hydrochloric acid.
7. Rinse the pipette well by drawing up the acid in the tube several times.
8. Let stand at least 30 minutes to permit the complete change of hemoglobin to acid hematin.
9. Place the acid hematin solution in the right-hand cup.
10. Fill the left-hand cup with distilled water and adjust the depth approximately to the reading expected. For normal blood about 9.
11. Secure a match of colors by adjusting the depth of the acid hematin solution. If this does not correspond with the depth of the distilled water, readjust both the distilled water and acid hematin solution until the colors match at the same depth in each cup.
12. Take the colorimeter reading and determine the number of grams of hemoglobin from the conversion table that comes with the Newcomer filters.

III. Photoelectric Colorimeter Methods.

A. *Oxyhemoglobin.*

1. Place 10 cc. of distilled water in a colorimeter tube.
2. Obtain a large drop of blood and draw it into a Sahli pipette exactly to the 20 c. mm. mark (0.02 cc.).
3. Wipe blood off the outside of the pipette and blow its contents into the distilled water, rinsing the pipette well by drawing up the water in the tube several times.
4. When ready to read in the photoelectric colorimeter, adjust the galvanometer to 100 using filter No. 540 and a tube containing distilled water as the blank.
5. Add 1 drop of ammonium hydroxide to the tube containing the blood, mix by inversion, and read immediately.
6. Obtain the hemoglobin content for the galvanometer reading from the table of values.
7. Calculation for the Evelyn instrument:

$$X = \frac{100\,L}{K_2}$$

X = gm. of hemoglobin per 100 cc. of blood.
$L = 2 -$ log. of the galvanometer reading.
$K_2 = 2.58$ which has been calculated for this method using the Evelyn instrument.

8. *Calibration of a Standard Curve for other Instruments.*

a. Most photolometers are calibrated for hemoglobin determinations when received.
b. If not, a calibration curve may be prepared from samples of whole blood. The amount of hemoglobin of each sample must be accurately determined by either Van Slyke's method for oxygen capacity or Wong's method for iron content.
c. Make the following dilutions from a stock solution of 10 cc. of blood diluted to 100 cc. with distilled water.

10% solution of blood	Distilled water	Blood content
1 cc. +	19 cc. =	0.005 cc.
1 cc. +	9 cc. =	0.010 cc.
3 cc. +	17 cc. =	0.015 cc.
2 cc. +	8 cc. =	0.020 cc.
5 cc. +	15 cc. =	0.025 cc.
3 cc. +	7 cc. =	0.030 cc.

d. Adjust the galvanometer to 100 with a tube containing distilled water (blank).
e. Add one drop of ammonium hydroxide to 10 cc. of each of the above dilutions, invert to mix, and read immediately.
f. Repeat, making new dilutions from the 10% solution of blood.
g. Repeat using at least 2 other samples of blood for stock solutions.
h. Make a curve on semilogarithmic graph paper using the average of the galvanometer readings for each solution.
i. List in a table the values (gm. of Hgb. per 100 cc. of blood) for each division on the galvanometer.

B. *Total Hemoglobin* (Evelyn and Malloy's Method).

Principle: Oxyhemoglobin is converted to methemoglobin by the use of potassium ferricyanide. Other forms of hemoglobin, such as methemoglobin, nitric oxide hemoglobin, and any other extraneous blood pigment which absorbs light at a wave length of 620 millimicrons, are quantitatively changed to cyanmethemoglobin by the use of potassium cyanide.

1. *General Considerations.*
   a. The error of the test is not more than ±0.2 gm. per 100 cc. of blood.
   b. This method is used to determine the presence of some abnormal types of hemoglobin.
      1) Determine the oxyhemoglobin content of the blood. The difference between this and the total hemoglobin is the amount of other hemoglobin present.
      2) The method does not differentiate between the types of hemoglobin. Carbon monoxide hemoglobin is not determined because it has same wave length as oxyhemoglobin.
      3) Carbon monoxide can be easily detected by the following simple test.
         a) Place 3 cc. of distilled water in a test tube, add 3 to 5 drops of blood, and then add 1 drop of a 5% sodium hydroxide solution.
         b) Mix gently and let stand 1 hour.
         c) Normal blood gives a greenish-brown color, carbon monoxide blood more or less pink.
         d) Always run a control using normal blood.
   c. If sulfhemoglobin is present, the total hemoglobin will be a higher value than the oxyhemoglobin, but the amount of increase is only a small portion of the sulfhemoglobin present.
   d. It is necessary to have 5 gm. of reduced hemoglobin and 3 gm. of methemoglobin per 100 cc. of blood to produce cyanosis.

2. *Method.*
   a. Determine the oxyhemoglobin by the method above simultaneously with the following procedure.
   b. Place 10 cc. of freshly prepared M/60 phosphate buffer solution in each of 2 colorimeter tubes (label 1 and 2).
   c. Obtain and deliver 0.02 cc. of whole blood into tube 2 as described under the method for oxyhemoglobin.
   d. Mix and let stand 2 minutes.
   e. Add 1 drop of 20% potassium ferricyanide to each tube (1 and 2), mix, and let stand 10 minutes.
   f. Add 1 drop of 5% potassium cyanide to each tube, mix, and let stand 2 minutes.
   g. Add 1 drop of ammonium hydroxide to each tube and mix.
   h. Immediately adjust the galvanometer to 100 using filter No. 540 and tube 1 as the blank; then read tube 2.
   i. Calculation for the Evelyn instrument:

$$X = \frac{100\,L}{K_2}$$

X = gm. total hemoglobin per 100 cc. of blood.
$L = 2 -$ log. of the galvanometer reading.
$K_2 = 2.40$ which has been calculated for this method using the Evelyn instrument.
Total hemoglobin — oxyhemoglobin = abnormal form of hemoglobin present.

3. *Calibration of a Standard Curve for other Instruments.*
   a. Make stock solutions of blood and dilutions as described under the oxyhemoglobin method but use M/60 phosphate buffer instead of distilled water.
   b. Use 10 cc. of the dilutions and proceed as described above for total hemoglobin.
   c. Make a table of values for gm. of total hemoglobin per 100 cc. of blood for each galvanometer division.

4. *Solutions.*
   a. *M/15 Phosphate buffer*—pH 6.
      1) Prepare phosphate buffer solutions as described on page 259.
      2) Mix according to pH 6 in Table 79, page 260.
   b. *M/60 Phosphate buffer*—pH 6.
      1) Pipette 25 cc. of the M/15 phosphate buffer solution into a 100 cc. volumetric flask and dilute to volume with distilled water.
      2) Prepare fresh before each determination.
   c. *Potassium ferricyanide solution—20%.*
      1) Place 10 gm. of potassium ferricyanide [$K_3Fe(CN)_6$] in a 50 cc. volumetric flask and dissolve in 35 cc. of warm distilled water.
      2) Cool and dilute to volume with distilled water.
   d. *Potassium cyanide solution—5%.*
      1) Place 2.5 gm. of potassium cyanide (KCN) in a 50 cc. volumetric flask and dissolve in about 30 cc. of distilled water.
      2) Dilute to volume with distilled water.

5. *Interpretation of Total Hemoglobin Findings.*
   a. Total hemoglobin should be the same value as the oxyhemoglobin of a normal individual.
   b. Increased above oxyhemoglobin in:
      1) Nitric oxide poisoning.
      2) After certain drugs producing methemoglobin.
      3) When blood contains sulfhemoglobin.

IV. Direct Matching Methods for Hemoglobin.

A. ***Tallqvist's Method.***

1. A Tallqvist's scale is used.
2. Take up a large drop of blood slowly with the absorbent paper.
3. Fold paper so that a part of the paper forms a background for the drop of blood.
4. Just as the gloss is leaving, before the air has darkened the hemoglobin, compare with the color scale.
5. The light should fall directly on the standard and the sample of blood.
6. Report the per cent of the color scale that matches the blood, estimating the per cent when the color of the blood falls between two of the standards; 100 per cent corresponds to 15.8 gm. of oxyhemoglobin in 100 cc. of blood.

B. ***Dare's Method.***

1. A Dare hemoglobinometer is used.
2. The blood is allowed to flow by capillarity into the slit between the 2 plates of glass clamped in the small metal holder.
3. One of the pieces is milk glass, the other clear.
4. This holder is then placed in the instrument with the milk glass toward the source of light.
5. By looking through the tube, compare with different portions of the circular glass disc of graduated thickness which is revolved by means of the small wheel at the top of the instrument.
6. Turn the wheel until the 2 colors are identical, then read the per cent or grams of hemoglobin at the arrow on the side of the instrument. This instrument tends to read a little too low in the upper part of the scale.
7. The entire operation, from the puncturing of the patient to the cleaning of the holder after the reading, should take not more than 2 minutes or the result will be incorrect.
8. Readings of 100 per cent on the scale correspond to 16.0 grams of oxyhemoglobin in each 100 cc. of blood.

V. Interpretation of Hemoglobin.

A. ***Normal Values***—vary according to age, sex, and locality.

1. Men: 14-18 gm. per cent.
2. Women: 12-16 gm. per cent.
3. Normal women in late pregnancy: 8.5-14 gm. per cent.
4. Children—see Table 6.

B. ***Abnormal Values.***

1. *Increased* (hyperchromemia) in polycythemia, dehydration, in poorly compensated heart disease with cyanosis, and in changing from low to high altitude.
2. *Decreased* (oligochromemia) in anemias.

TABLE 6. NORMAL BLOOD COUNT IN CHILDREN

Age	Hemoglobin gm. per 100 cc.	Erythrocytes mill. per c. mm.	Leukocytes thousands per c. mm.	Granulocytes per cent	Lymphocytes per cent	Monocytes per cent
Birth	18 - 26.5 Mean - 22.2	4.76 - 6.95* Mean - 6.28	20 - 30	50 - 75	20 - 25	5 - 15
2 weeks......	13.4 - 19.2 Mean - 16.1	4.32 - 6.14 Mean - 5.55	10 - 18	40 - 45	40 - 45	4 - 8
1 month.....	12.1 - 17.3 Mean - 15.4	4.08 - 6.05 Mean - 5.25	8 - 15	30 - 40	45 - 60	2 - 6
3 months....	9.8 - 16.2 Mean - 11.9	3.65 - 5.22 Mean - 4.55	8 - 14	30 - 40	45 - 60	2 - 6
6 months....	10.6 - 15.4 Mean - 12.7	3.87 - 5.39 Mean - 4.83	8 - 14	30 - 40	45 - 60	2 - 6
1 year.......	9.6 - 14.9 Mean - 11.9	3.95 - 5.32 Mean - 4.63	6 - 12	30 - 40	45 - 60	2 - 6
2 years......	9.7 - 14.2 Mean - 11.6	3.95 - 5.26 Mean - 4.59	6 - 12	35 - 45	40 - 55	2 - 6
4 years......	9.41 - 14.3 Mean - 11.7	4.11 - 5.59 Mean - 4.89	6 - 12	40 - 50	40 - 45	2 - 6
6 years......	10.5 - 13.8 Mean - 11.7	4.19 - 5.96 Mean - 4.97	6 - 10	45 - 55	35 - 40	2 - 6
8 years......	10.1 - 13.3 Mean - 11.9	4.43 - 6.02 Mean - 5.10	6 - 10	50 - 65	20 - 35	2 - 6
10 years......	10.1 - 14.3 Mean - 12.4	4.38 - 6.16 Mean - 5.05	6 - 10	50 - 60	25 - 35	2 - 6
12 years......	11.0 - 13.5 Mean - 12.5	4.47 - 5.80 Mean - 5.17	6 - 10	50 - 60	25 - 35	2 - 6

*200 to 2000 are nucleated.

C. ***Hemoglobinemia*** is the presence of free hemoglobin in the blood plasma.

1. Found in severe infections, severe burns and frost bite, poisoning with potassium chlorate and mushrooms, and in paroxysmal hemoglobinuria and hemolytic blood transfusion reactions.
2. *Hemoglobinuria* is the presence of free hemoglobin in the urine and occurs when the free hemoglobin in the plasma reaches a concentration between 30 and 300 mg. (average 150 mg.) per 100 cc.

Indices, Cell Pack, and Total Blood Volume

It is very important in calculating all indices that accurate hemoglobin determinations and erythrocyte counts be made, as a slight error in either will give incorrect indices and result in a false interpretation.

I. Hemoglobin Content per Erythrocyte.

A. *Color Index (C.I.).*

1. This index indicates the amount of hemoglobin in the average erythrocyte compared with the amount in a normal erythrocyte.
2. The average normal hemoglobin for blood with 5 million erythrocytes per c. mm. is 14.5 gm. per 100 cc. of blood.
3. The color index is calculated as follows:

$$\text{C.I.} = \frac{\dfrac{\text{gm. Hgb.}}{14.5}}{\dfrac{\text{R.B.C. in mill.}}{5.0}} \quad \text{or}$$

$$\text{C.I.} = \frac{\text{gm. Hgb.}}{\text{R.B.C. in mill.}} \times 0.345$$

4. Use nomogram (Fig. 4) for obtaining value.
5. When hemoglobin is expressed in per cent, a rough estimation can be obtained by dividing the percentage of hemoglobin by the first 2 figures of the erythrocyte count multiplied by 2. It is important to remember that 100 per cent on different instruments represents a different number of grams of hemoglobin per 100 cc. of blood.
6. The normal color index varies from 0.90 to 1.10.
7. Refer to Table 7 (p. 44) for values in anemias.

B. *Mean Corpuscular Hemoglobin (M.C.H.) in micromicrograms* is the weight of the hemoglobin in the average erythrocyte.

$$\text{M.C.H.} = \frac{\text{gm. Hgb.} \times 10}{\text{R.B.C. in mill.}}$$

1. Use nomogram (Fig. 5) for obtaining value.
2. Normal values range from 27 to 32 micromicrograms (average 29.5).
3. See Table 7 (p. 44) for values in anemias.

II. Volume of Erythrocytes.

A. *Cell Pack (Cell Volume Per Cent).*

1. From the hematocrit reading the percentage of packed erythrocytes in whole blood (cell volume per cent) is determined.
2. Withdraw 5 cc. of venous blood in a dry syringe and place in a bottle containing the exact amount of dried ammonium and potassium oxalate anticoagulant solution for the amount of blood (p. 34).
3. Shake the bottle immediately for a few seconds to prevent clotting and shake for 3 minutes when ready to fill the hematocrit tube.
4. Place the blood in a Wintrobe hematocrit tube by means of a capillary pipette, the tip of which is at first inserted to the bottom of the tube and then slowly withdrawn until the tube is filled to the 10 mark.
5. Stopper to prevent evaporation (this is not necessary if the tube is filled exactly to the 10 mark).
6. Centrifuge for 30 minutes at 3000 revolutions per minute.
7. The proportion of erythrocytes to the total volume of blood is read from the scale which reads from the bottom upward without including the buffy layer of leukocytes in the reading.
8. Multiply by 10 for volume per cent.
9. If the blood is above the 10 mark, calculate the cell pack by dividing the height of the column of packed erythrocytes by the total height of the column of cells and plasma and multiply the result by 100.
10. *Note the following:*
    a. *Color and opacity of plasma.*
        1) Yellow in jaundice.
        2) Milky in lipemia.
        3) Cloudy in multiple myeloma.
    b. *Reddish-gray layer of packed leukocytes and platelets* (buffy layer).
        1) Normally 0.5 to 1 mm. in thickness.
        2) Each 0.1 mm. is equivalent to 1000 leukocytes per c. mm.
        3) Platelets are on upper part of layer.
11. *Cleaning hematocrit tube.*
    a. Attach a capillary pipette to a suction pump and place the tip through the blood to the bottom of the tube.
    b. Turn the suction pump on and hold the tube under running water.
    c. The suction not only withdraws blood from the tube but draws water into the instrument.
    d. When clean, fill with cleaning solution

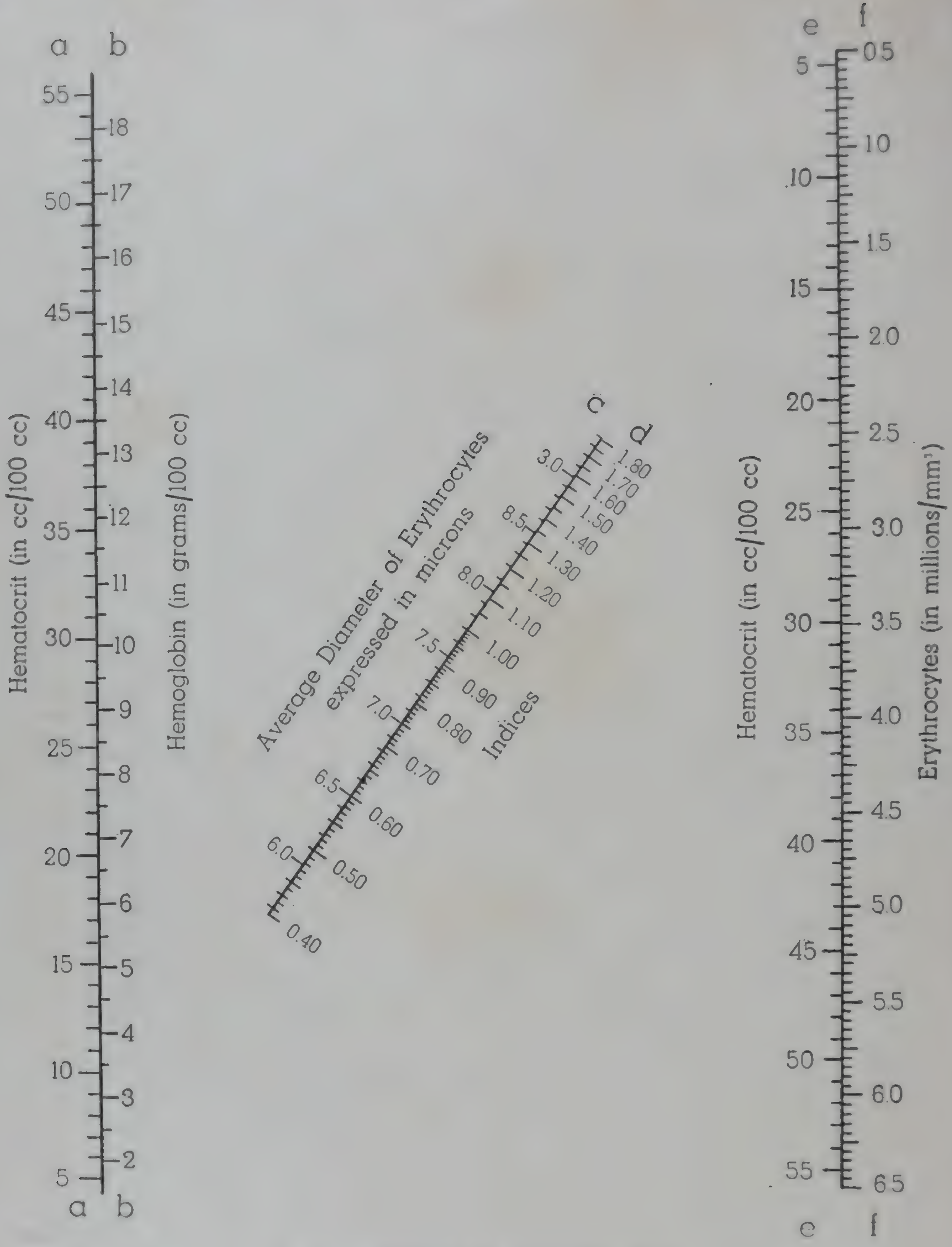


FIG. 4. NOMOGRAM FOR CALCULATING INDICES.

Color Index	apply scale b. d. f.
Volume Index	apply scale a. d. f.
Saturation Index	apply scale b. d. e.
Average Diameter of Erythrocytes	apply scale a. c. f.

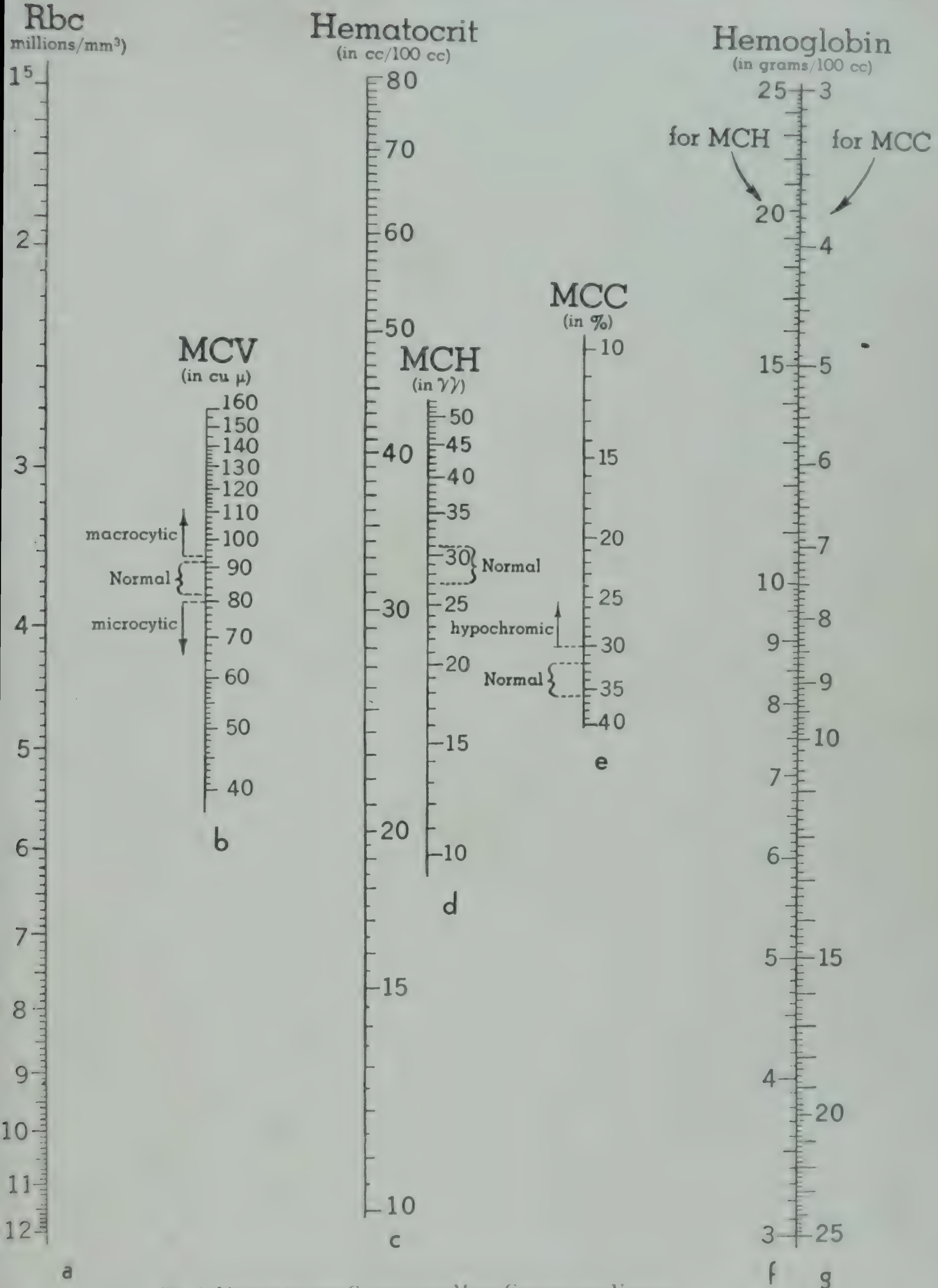


Fig. 5. Nomogram for Calculating Mean Corpuscular Values.
Mean Corpuscular Volume (M. C. V.) . . . . apply scale a. b. c
Mean Corpuscular Hemoglobin (M. C. H.) . . . apply scale a. d. f
Mean Corpuscular Hbg. Conc. (M. C. H. C.) . . . apply scale c. e. g
(From Wintrobe's "Clinical Hematology," courtesy of Lea and Febiger, Philadelphia

and let stand overnight; rinse thoroughly first with tap water and then distilled water.

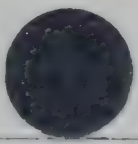
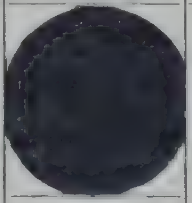
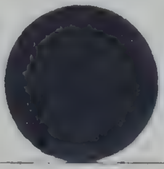
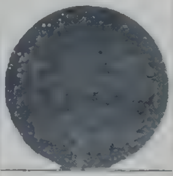
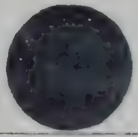
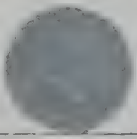
e. Invert the tube to dry or dry with acetone.

12. *Normal Values for Cell Pack.*
    a. Men: 40-54%, average 47%.
    b. Women: 37-47%, average 42%.
    c. Average value for 5 million erythrocytes is 43%.
    d. Normal women in late pregnancy: 23-34%.

B. Volume Index (V.I.).

1. This index indicates the mean volume of the average erythrocyte compared with the mean volume of the normal erythrocyte.

TABLE 7. HEMOGLOBIN CONTENT, VOLUME, AND SIZE OF ERYTHROCYTES IN ANEMIAS

*Classification of Anemias	Normocytic Normochromic Blood	Macrocytic Hyperchromic Anemia	Macrocytic Normochromic Anemia	Macrocytic Hypochromic Anemia	Normocytic Normochromic Anemia	Normocytic Hypochromic Anemia	Microcytic Hypochromic Anemia
Relative Volume and Hemoglobin Content of Corpuscle							
Erythrocytes (mill.)	4.5 - 5.5	1.0 - 2.5	2.0 - 3.5	2.0 - 3.5	2.5 - 4.5	3.0 - 4.5	3.0 - 4.5
Hemoglobin (gm.)	14 - 16	6 - 8	6 - 8	5 - 7	8 - 12	5 - 8	4 - 8
Color Index	0.90 - 1.10	1.20 - 2.0	0.90 - 1.10	0.70 - 0.90	0.90 - 1.10	0.50 - 0.80	0.40 - 0.80
Mean Corpuscular Hgb. (micromicrograms)	27 - 32	30 - 52	27 - 32	20 - 26	27 - 32	20 - 26	14 - 21
Volume Index	0.90 - 1.10	1.20 - 2.0	1.20 - 1.60	1.20 - 1.60	0.90 - 1.10	0.90 - 1.10	0.70 - 0.90
Mean Corpuscular Volume (c. microns)	82 - 92	95 - 160	95 - 140	95 - 140	80 - 94	72 - 79	50 - 71
Saturation Index	1.0	0.90 - 1.0	0.70 - 0.90	0.60 - 0.80	0.90 - 1.0	0.50 - 0.80	0.60 - 0.90
Mean Corpuscular Hgb. Conc. (%)	32 - 36	31 - 36	31 - 36	25 - 33	32 - 36	31 - 36	21 - 29
Mean Corpuscular Diameter (microns)	6.7 - 8.0	7.5 - 9.6	7.0 - 9.0	7.0 - 9.0	6.7 - 8.0	6.5 - 8.5	5.8 - 7.5

*See blood findings in disease, page 95.

2. The cell pack and erythrocyte count must be determined.
3. The volume index is calculated as follows:

$$V.I. = \frac{\frac{\text{Cell Pack}}{43}}{\frac{\text{R.B.C. in mill.}}{5.0}} \quad \text{or}$$

$$V.I. = \frac{\text{Cell Pack}}{\text{R.B.C. in mill.}} \times 0.116$$

4. Use nomogram (Fig. 4, p. 42) for obtaining value.
5. The normal volume index varies from 0.90 to 1.10.
6. See Table 7 for values in anemias.

C. Mean Corpuscular Volume (M.C.V.) in cubic microns is the mean volume of the average erythrocyte.

$$M.C.V. = \frac{\text{Cell Pack} \times 10}{\text{R.B.C. in mill.}}$$

1. Use nomogram (Fig. 5, p. 43) for obtaining value.
2. Normal values range from 82 to 92 cubic microns (average 87).
3. See Table 7 for values in anemias.

III. Hemoglobin per Unit Volume of Erythrocytes.

A. Saturation Index (S.I.).

1. This index indicates the concentration of hemoglobin per unit volume of erythrocytes relative to normal.
2. The color and volume indices must be known or the cell pack and grams of hemoglobin must be determined.
3. The saturation index is calculated as follows:

$$S.I. = \frac{\frac{\text{gm. Hgb.}}{14.5}}{\frac{\text{Cell Pack}}{43}} \quad \text{or}$$

$$S.I. = \frac{\text{Color Index}}{\text{Volume Index}}$$

4. Use nomogram (Fig. 4, p. 42) for obtaining value.

The normal saturation index is 1, it is never greater than 1.

See Table 7 for values in anemias.

B. ***Mean Corpuscular Hemoglobin Concentration (M.C.H.C.) in per cent*** is the concentration of hemoglobin in the average erythrocyte; that is, the ratio of the weight of hemoglobin to the volume in which it is contained.

$$\text{M.C.H.C.} = \frac{\text{gm. Hgb.} \times 100}{\text{Cell Pack}}$$

Use nomogram (Fig. 5, p. 43) for obtaining value.

2. Normal values range from 32 to 36 with an average of 34 per cent.

See Table 7 for values in anemias.

IV. Diameter, Thickness, and Volume Thickness Index of the Erythrocyte.

A. ***Mean Corpuscular Diameter (M.C.D. or Price-Jones Count)*** **in microns.**

1. An ocular micrometer in which one division equals 1 micron is necessary.
2. Place the micrometer with the ruled surface down on the diaphragm of the eyepiece of a microscope.
   a. The micrometer must be standardized with the eyepiece, oil immersion lens, and tube length of the microscope to be used.
   b. With a stage micrometer measure the distance between the lines on the ocular micrometer which may be more or less than one micron for that particular microscope.
3. Place a thin film of blood stained with Wright's stain under the oil immersion lens and measure 100 consecutive erythrocytes to the nearest 0.5 micron. Do not measure any cell that is not round.

Move to another part of the film and measure another 100 consecutive cells.

Tabulate the cells counted into groups which differ by 0.5 micron and plot a curve. See Fig. 6.

Calculation of Mean Corpuscular Diameter.
   a. Multiply the number of cells in each group by the diameter of that group.
   b. The sum of the products obtained in (a) divided by the total number of cells counted will give the mean corpuscular diameter.

Normal values range from 6.7 to 8 microns (average 7.5).

See Table 7 and Fig. 6 for values in anemias.

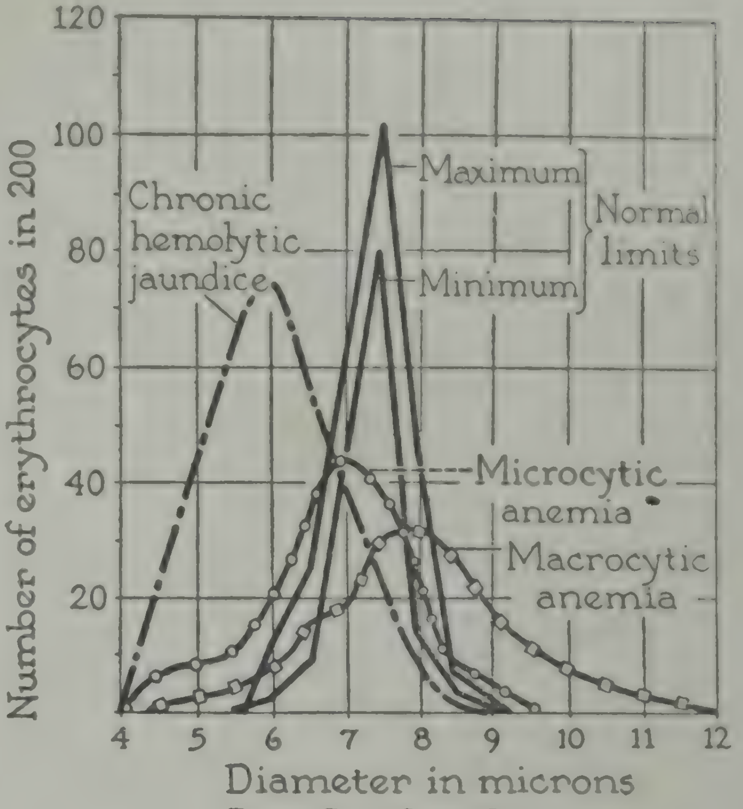


FIG. 6. PRICE-JONES CURVE.

B. ***Mean Corpuscular Thickness (M.C.T.) in microns*** can be determined by the following formula:

$$\text{M.C.T.} = \frac{4\,\text{V}}{\text{D}^2 \times 3.1416}$$

V = Mean Corpuscular Volume.
D = Mean Corpuscular Diameter.

1. Normal average thickness of an erythrocyte is 2.0 microns.
2. Average thickness in various anemias.
   a. Simple microcytic anemia—1.60 microns.
   b. Macrocytic normochromic anemia—2.20 microns.
   c. Obstructive jaundice—1.60 microns.
   d. Chronic hemolytic jaundice — 3.02 microns.

C. ***Volume Thickness Index (V.T.I.)*** is a ratio of the volume of the erythrocyte to a calculated volume for the measured diameter of the erythrocyte.

$$\text{V.T.I.} = \frac{\text{Volume Index}}{\text{Calculated Volume Index}}$$

1. The calculated volume index is obtained from Fig. 4, page 42. It is the figure in column D corresponding to the measured mean corpuscular diameter (Price-Jones) in column C.
2. The normal volume thickness index is 1.
3. This index remains practically normal in all anemias except chronic hemolytic jaundice in which it ranges from 1 to 3.5.

V. Total Blood and Plasma Volume.

A. ***Principle:*** A dye must be used which is nontoxic and nonhemolytic, disappears slowly from the blood stream, is found only in the plasma (not in the cells), and retains its color after entering the blood stream. The dilution of the dye in the circulating blood plasma is measured and with the cell pack the circulating volume of blood is calculated.

B. ***General Considerations.***

1. Patient must be in a fasting state, have rested lying down for 20 minutes before injecting the dye, and remain lying down until the last sample of blood is obtained.
2. Use only dry syringes and needles, taking all precautions to avoid hemolysis.
3. Do not use a tourniquet while withdrawing blood.
4. No dye must be lost in the tissues during injection.
5. A stop watch should be used for timing the injection of dye and the withdrawal of blood.
6. It takes from 4 to 6 minutes for the dye to become thoroughly mixed with the blood; therefore, the blood should not be withdrawn for at least 4 minutes.

C. ***Congo Red Method.***

1. See general considerations (B).
2. Weigh the patient and calculate the number of cc. of a 1.5 per cent sterile solution of Congo red to be injected by using the following formula which has been determined by experiment:

$$\frac{\text{Weight in lbs.}}{8.8} = \text{cc. to be injected.}$$

3. Withdraw 10 cc. of blood by venipuncture and place in a bottle containing the correct amount of dry ammonium and potassium oxalate and shake. Label A.
4. Without removing the needle from the vein, change syringes and inject the proper amount of dye which should not exceed 18 cc. Draw blood back into the syringe 3 to 5 times to wash out the residual dye.
5. In 4 minutes from the beginning of the injection, withdraw 10 cc. of blood from the opposite arm, using a clean dry syringe. Place the blood in another bottle containing the correct amount of dry oxalate and shake. Label B.
6. Determine the cell pack of blood A.
7. Place bloods A and B in each of 2 tubes and centrifuge.
8. To prepare the standard, mix 2 cc. of plasma A, 2 cc. of a 1-200 dilution of the same Congo red solution used for injection, and 4 cc. of 0.85% NaCl solution.
9. To prepare the unknown plasma, mix 2 cc. of plasma B and 6 cc. of 0.85% NaCl solution.
10. Compare unknown plasma in the colorimeter with standard set at 10. This reading i R in the calculation.
11. *Calculations:*

$$\text{Plasma Volume in cc.} = \frac{\text{Dye injected (cc.)} \times 200 \times}{10}$$

$$\text{Total Blood Volume in cc.} = \frac{\text{Plasma volume (cc.)} \times 1}{\substack{100 - \text{cell pack} \\ \text{(plasma per cent)}}}$$

12. Divide the plasma volume and also the tot blood volume by the patient's weight in kilograms to determine the values of each pe kilogram.
13. *Normal Values.*
    a. Plasma volume is 35 to 50 cc. per kg.
    b. Total blood volume is 65 to 90 cc. per kg
    c. Total blood volume of males is abou 25% greater than females due chiefly t the higher red cell volume of the male while the plasma volume is approximatel the same in both sexes.

D. ***Evans Blue Dye Method.***

1. Evans Blue dye, T-1824 (E.K. 3873) is usec
2. *Dye solution* (0.1%).
    a. Weigh out 1 gram of dye on the analytical balance.
    b. Dissolve in triple distilled water and dilut to volume in a liter volumetric flask.
    c. Filter through a Jena or Pyrex sintere glass filter.
    d. The amount of dye left in the filter i insignificant and water should not be added in an effort to dissolve it.
    e. Place approximately 11 cc. portions o this solution in neutral glass ampules, seal and immediately autoclave at 121°C. fo 20 minutes.
3. *Calibration on the Photoelectric Colorimeter*
    a. Place exactly 10 cc. of the dye solution from an ampule in a 100 cc. volumetri flask, dilute to volume with 0.85% NaC solution, and mix.
    b. Transfer 1, 2, 3, 4, 5, and 6 cc. of this dye solution to test tubes and dilute each t 10 cc. with 0.85% NaCl solution.
    c. Mix each thoroughly.
    d. Use pooled serum (40 cc.) free fron hemolysis of erythrocytes for the remaining dye solutions and blank.
        1) The total protein and albumin content of the pooled serum must be within th normal range because the quantity o protein present affects the photoelectri properties of the dye.
        2) If the dye is used in cases presenting extreme pathologic changes in the composition of the blood, the dye solution

should be standardized with serum from the patient in question.

e. Transfer 0.6 cc. of each dilution of dye to colorimeter tubes, add 5.4 cc. of the pooled serum to each, and mix.

f. Prepare a blank consisting of 5.4 cc. of pooled serum and 0.6 cc. of 0.85% NaCl solution.

g. Adjust the galvanometer to 100 with the blank using filter No. 635 and then read each dilution of the dye.

h. Repeat 3 or 4 times; then prepare a curve on semilogarithmic graph paper using the corresponding amounts of standard dye solution: 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg. per cent plotted against the average of the galvanometer readings.

i. Make a table of the values in mg. per cent of dye for each division on the galvanometer.

Procedure.

a. See general considerations (B).

b. Withdraw 20 cc. of blood from a vein; place 17 cc. in a test tube to clot and 3 cc. in a bottle containing the correct amount of dry ammonium and potassium oxalate and shake. Label A. (Plasma may be used instead of serum.)

c. Without withdrawing the needle, inject exactly 10 cc. of the dye solution with another syringe.

d. The injection should take 30 seconds; draw blood back into the syringe about 3 times and reinject to wash out the residual dye in the syringe.

e. In exactly 10 minutes from the beginning of the injection, withdraw 20 cc. of blood from a vein in the opposite arm and place in a test tube to clot. Label B.

f. Do a cell pack on the oxalated blood.

g. Centrifuge the clotted blood samples A and B and remove the serum, then recentrifuge the serum so that it is perfectly clear.

h. Place 6 cc. of serum A and serum B in separate colorimeter tubes and read in the photoelectric colorimeter using filter No. 635. Use serum A as the blank to set the galvanometer at 100.

i. *Calculation:*

   1) Determine the mg. per cent of dye in the serum from the table of values.

   2) A correction of the concentration of dye in the blood must be made for the amount of dye absorbed by the tissues during the period from the injection of the dye to the withdrawal of blood. Add 1% of the mg. per cent of dye in the blood for the 10 minute period (and 0.1% for each additional minute if the blood is not obtained at exactly 10 minutes).

   3) Plasma Volume in cc. =

$$\frac{10(\text{mg. of dye injected}) \times 100}{\text{Conc. of dye in serum (corrected)}}$$

   4) Total Blood Volume in cc. =

$$\frac{\text{Plasma volume (cc.)} \times 100}{100 - \text{cell pack}}$$

   5) Divide the plasma volume and the total blood volume by the patient's weight in kilograms to determine the value for each per kilogram of body weight.

5. *Normal Values.*
   a. Plasma volume for men 40 to 48 cc. per kg.; for women 37 to 46 cc. per kg.
   b. Total blood volume for men 70 to 85 cc. per kg.; for women 59 to 73 cc. per kg.

E. *Interpretation of Blood Volume Findings.*

1. *Increased Blood Volume.*
   a. *Due to increase in number of cells:*
      Cardiac decompensation
      Erythremia (polycythemia vera)
      Leukemia (W.B.C.)
   b. *Due to increase in plasma:*
      Cirrhosis of the liver
      Scurvy
      Splenomegaly
   c. *Due to increase in cells and plasma:*
      Exposure to moderate heat
      Hyperthyroidism
2. *Decreased Blood Volume.*
   a. *Due to decrease in number of cells:*
      Chronic nephritis
      Pernicious anemia
   b. *Due to decrease in plasma:*
      Dehydration due to severe burns, Asiatic cholera, exposure to severe heat, and artificial fever.
      Prolonged exposure to cold
      Exposure to high altitudes
      Surgical shock
      Addison's disease
      Diabetic acidosis
      Erect posture
   c. *Due to decrease in cells and plasma:*
      Hemorrhage
      Myxedema (mostly cells)

Preparation and Staining of Blood Smears

I. Preparation of Blood Smears.

A. *Slide Method.*

1. Before using glass slides, clean with alcohol,

rinse in warm water, and wipe dry with a clean lint-free towel or gauze.

2. Touch a perfectly clean grease-free slide to a small drop of blood, 2 mm. in diameter, taking care that the slide does not touch the skin. (The drop of blood should be placed about 2 cm. from one end of the slide.)
3. Place slide on a table and immediately place the end of another slide (spreader slide) against the surface of the first, holding it at an angle of 45° or more. (The angle of the spreader is important because this angle prevents the leukocytes from bunching along the edges.)
4. Draw it back against the drop of blood which will spread across the surface between the 2 slides. See Fig. 7.
5. Push the spreader slide slowly and steadily across the first and the blood will follow, making an even film.
6. The thickness of the film can be varied by the rapidity with which the slide is pushed; the slower the motion, the thinner the smear. A thin smear is essential.
7. Dry rapidly by waving in the air; do not dry with heat.
8. When dry, label with patient's name by writing with pencil on the thick end of the blood smear. See Fig. 8.
9. Lay slide with smeared surface down to protect from dust and flies.

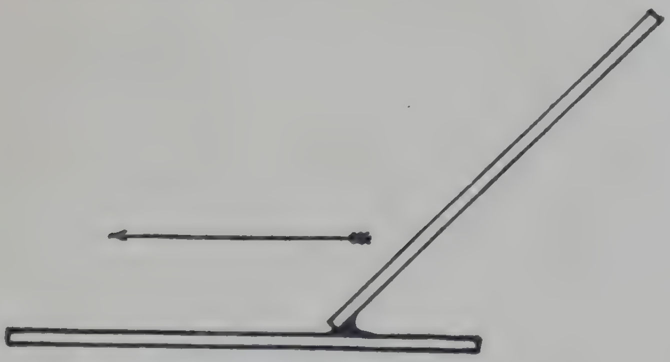


Fig. 7. Preparation of a Blood Smear.

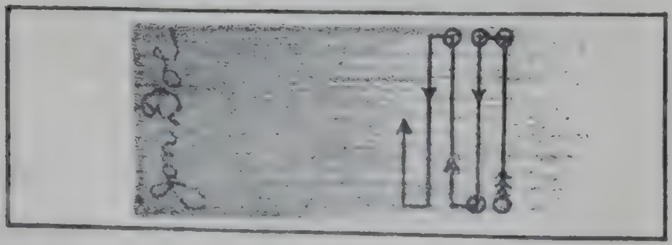


Fig. 8. Satisfactory Thin Blood Smear, Method of Labeling and Examining Smear.

10. *Criteria of a Good Smear.*
    a. Must have a smooth even surface, free from ridges, waves, and holes.
    b. Smear should not extend to the edges or to end of slide but should be at least 1 to 2 inches long.
    c. The leukocytes must not be bunched a the edges or at the end.
    d. More than one half of the smear shoul be thin enough that the erythrocyte just touch each other but do not overla
    e. The size of the cells is influenced by th thickness of the smear.
        1) The thinner the smear the larger th cells.
        2) For correct identification of cells, i is necessary to have a thin smear.

B. *Slide and Cover Glass Method.*

1. Touch a clean grease-free slide to a small dro of blood, 2 mm. in diameter, so it is about inch from the end and near the edge of th slide.
2. Apply a clean No. 2 cover glass (22 x 22 mm. over the drop.
3. As soon as the blood has ceased spreadin underneath the cover glass, place the tips o 2 fingers on the cover and with the lightes possible pressure quickly slide the cover glas on the slide and off the edge opposite th drop of blood.
4. This leaves a thin film of blood with a good distribution of cells.

C. *Cover Glass Method.*

1. Use No. 1 cover glasses (22 x 22 mm.) tha have previously been cleaned in concentrated nitric acid, washed with hot water, and kept in alcohol until used.
2. Dry 2 cover glasses with a clean, lint-free towel or gauze.
3. One is held in each hand with the thumb and forefinger at adjacent corners.
4. A small drop of blood is touched with the cover glass held in the right hand.
5. This cover glass is quickly placed on top of the cover glass in the left hand.
6. After the blood has spread by capillary action, the cover glasses are drawn apart with a steady motion, care being taken to keep them parallel.
7. The drop of blood must be just large enough to cover the cover glass when spread.
8. Dry film in air.
9. Support cover glass on a cork smaller than the cover glass and stain.

II. Staining of Smears.

A. *Wright's Stain.*

1. See page 356 for preparation of Wright's stain and buffer solution.
2. Smears should be stained on the same day

they are made.

Cover the dried blood smear with Wright's stain and let stand for 2 minutes. The stain must not precipitate on the slide through evaporation.

Add an equal amount of buffer solution (pH 6.4), distributing it over the whole slide; none should run off the edges.

The stain and buffer may be evenly mixed by blowing gently on the surface. The mixture should show a brassy sheen.

Let stand 5 minutes. (To find the best timing each new batch of Wright's stain must be tested with various lengths of staining.)

Wash off stain by running a stream of water directly into the center of slide to prevent precipitation of stain on the blood film; continue washing for 30 seconds.

Remove the film of stain from the back of the slide by wiping with a cloth.

Stand slide on end and let dry in the air.

It is very important that the slides be watched and timed so the stain does not precipitate.

If the stain should precipitate, cover slide with 95% alcohol and wash off immediately.

If the stain is not deep enough, the slide may be stained again.

Criteria of a Good Stain.

a. Must be free of precipitated stain.
b. The erythrocytes are orange or pink color; the granules of the neutrophils lilac, of the eosinophils bright red, of the basophils deep blue; and the platelets are purplish blue with a distinct architecture.
c. If the stain is too acid, the erythrocytes are a bright red and the nuclei of the leukocytes pale blue to colorless.
d. If the stain is too basic, the erythrocytes are a slate blue.

Peroxidase Stain.

See page 356 for preparation of Washburn's stain.

Smear must be stained within 3 or 4 hours after being made.

Cover the dried blood smear with Washburn's stain and let stand 2 minutes.

Add an equal amount of freshly diluted hydrogen peroxide to the stain and let stand 3 to 4 minutes. (To make the diluted hydrogen peroxide add 6 drops to 25 cc. of distilled water.)

Wash thoroughly with tap water for 1 minute. (The slide can be dried and examined at once if time prevents counterstaining with Wright's stain.)

While still wet, flood with 95% alcohol and allow to stand 3 to 4 minutes, no longer.

7. Wash thoroughly with tap water and dry.
8. Flood with Wright's stain and allow to stand for 3 minutes.
9. Add twice the amount of buffer solution and allow to stand for 30 to 45 minutes. The slide must have a large amount of stain and buffer solution on it to prevent precipitation of stain. Leukemic bloods require 40 to 45 minutes.
10. Wash briefly with tap water, flood with 95% alcohol for 3 to 5 seconds, and immediately wash with tap water for 10 to 15 seconds.
11. Dry and examine.
12. All cells of the granulocyte series except myeloblasts give the peroxidase reaction; that is, the granules stain deep blue to black. Cells in the lymphocytic series do not give the peroxidase reaction, while a few granules in the monocytes may give the reaction.
13. Differentiate 100 leukocytes and calculate the percentage of cells showing peroxidase positive granules.

C. ***McCord's Method for Staining Basophilic Aggregations*** (stippling of erythrocytes).

1. The blood smear must dry from 1 to 3 hours, but no longer.
2. Cover one half of the smear longitudinally with a strip of filter paper.
3. With a pipette or dropper, apply the minimum amount of methyl alcohol required to moisten the filter paper so that it clings to the slide.
4. Leave until dry and then remove the paper. The methyl alcohol fixes the blood on this portion of the slide.
5. Cover the slide with diluted Manson's methylene blue stain and leave for 10 minutes.
6. Wash slide thoroughly 3 or 4 times with distilled water.
7. Dry in the air and examine.
8. Place a Whipple eyepiece micrometer in the microscope. Instead of a micrometer, a piece of paper the size of the eyepiece with an opening 5 mm. square cut in the center may be placed in the eyepiece. This reduces the size of the field.
9. In the unfixed portion of the slide, count the basophilic aggregations in 10 consecutive fields in 2 parallel rows making a total of 20 fields.
10. In the fixed portion of the slide, count all the erythrocytes in 5 consecutive fields and multiply by 4. (The areas counted in both the fixed and unfixed portions of the smear should be directly opposite each other.)

11. The basophilic aggregations are expressed as a percentage of erythrocytes.
12. The outline of the cells in the unfixed portion of the slide are mere shadows and the basophilic aggregations show up as masses, strands, or reticulum taking a blue stain.
13. In the fixed portion the erythrocytes are a clear brilliant blue.
14. Normally 0.5 to 1.5 per cent of the erythrocytes show basophilic aggregations when stained by this method.
15. In lead poisoning 1.5 to 4 per cent of the erythrocytes show basophilic aggregations.
16. Diffuse polychromatophilia, punctate basophilia, and reticulocytes are stained by this method.
17. *Stock Manson's Stain.*

Borax (sodium borate)	2.5 gm.
Boiling distilled water	50 cc.
Methylene blue	1 gm.

Dissolve the sodium borate in the boiling distilled water; add the methylene blue and filter. Dilute before using.

18. *Diluted Stain*—add 1 cc. of freshly filtered stock stain to 20 cc. of a 0.3% solution of sodium chloride. (Must be made the day it is used.)

D. *Vital Staining for Reticulocytes.*

1. Reticulocytes stain only while the cells are alive and do not stain in a dried smear.
2. One of two types of staining solutions may be used.
    a. A 1% solution of brilliant cresyl blue in 0.85% NaCl solution. Warm slightly to dissolve stain and filter when cool.
    b. A 1% solution of brilliant cresyl blue in methyl alcohol. Filter before using.
3. *Wet Smear Method.*
    a. Filter a small drop of the saline dye solution on a slide and add an equal sized drop of blood from a prick of the finger or ear.
    b. Mix by stirring with a pin, then place a clean cover glass on the drop.
    c. Seal with vaseline.
    d. Let stand at least 10 minutes before counting.
    e. This method can also be used by allowing a few drops of the alcoholic dye solution to dry on a clean slide and then adding a drop of blood and cover glass. The slides with the dried stain can be kept and used at any time.
4. *Dried Smear Method.*
    a. Filter about 1 cc. of the saline dye solution in a small test tube.
    b. Fill a leukocyte pipette approximately half full of blood; then fill with staining solution.
    c. Shake to mix and let stand 10 minutes.
    d. Shake for 3 minutes, blow out 3 drops, and then make 4 thin smears as described under preparation of blood smear (slide method).
    e. Counterstain with Wright's stain, leaving the Wright stain on 1 minute and the buffer solution on 2 minutes.
    f. Dry in the air and examine.
5. *Counting Reticulocytes.*
    a. Place a Whipple eyepiece micrometer in the microscope. Instead of a micrometer, a piece of paper the size of the eyepiece with an opening 5 mm. square cut in the center may be placed in the eyepiece. This reduces the size of the field.
    b. Count 1000 erythrocytes noting the number of cells showing reticulum, which appears as blue dots, filaments, skeins, or wreaths.

$$\frac{\text{No. of reticulated cells}}{10} = \text{\% of reticulocytes.}$$

    c. The total number of reticulocytes per c. mm. of blood is obtained by making an erythrocyte count and multiplying the result by the number of reticulocytes counted and dividing by 1000.
6. Normal findings are 0.5 to 1.5 per cent or 25,000 to 75,000 per c. mm.
7. An increase is considered one of the early signs of regeneration of erythrocytes by the bone marrow.
8. A count less than 0.5 per cent indicates an abnormally slow rate of production of erythrocytes by the bone marrow.

Examination of Blood Smears

I. Method of Examination.

A. *Preliminary Examination.*

1. Look over slide with a high power objective to locate best area to count.
2. See criteria of a good smear, page 48.
3. See criteria of a good stain, page 49.

B. *Method of Making a Differential Count.*

1. Use the oil immersion lens for making the count.
2. Begin at the upper edge of the smear and move the slide down to the lower edge, marking down the type of each leukocyte that appears in the field.
3. When the lower edge is reached, move the slide sideways for a short distance, classifying

all the leukocytes seen while moving the slide, and then move it up. See Fig. 8, page 48.

The number of cells to be counted and classified should be determined by the total leukocyte count. The following is recommended:

For counts up to 10,000, classify 100 cells; if any abnormal cells are seen count 200.

For counts of 10,000 to 20,000, classify 200 cells.

For counts of 20,000 to 50,000, classify 300 cells.

For counts over 50,000, classify 400 cells.

Any abnormality in the appearance of the erythrocytes should be noted.

II. Differential Leukocyte Count.

For Development of Blood Cells see Fig. 9, page 57.

Schilling classifies the granular cells in groups according to their development. See Table 8.

TABLE 8. SCHILLING'S HEMOGRAM (MODIFIED)

	Leukocyte Count	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocytes (Juveniles)	Band (neutrophils with a single lobe nucleus), also called nonfilament.	Segmented (neutrophils with nuclei having two or more lobes), also called filament forms.	Eosinophils	Basophils	Lymphocytes	Monocytes
ormal per cent relative values		0	0	0	0 to 1	2 to 6	50 to 70	1 to 3	0 to 1	20 to 40	2 to 8
ormal absolute lues	4,000 to 10,000	0	0	0	0 to 100	80 to 600	2,000 to 7,000	40 to 300	0 to 100	800 to 4,000	80 to 800

Arneth classifies the neutrophils according to their age, that is, according to the number of lobes which their nuclei possess.

One round or indented nucleus (youngest cell), 5 per cent.

Two nuclear divisions, 35 per cent.

Three nuclear divisions, 41 per cent.

Four nuclear divisions, 17 per cent.

Five or more nuclear divisions (oldest cell), 2 per cent.

Significance of Schilling and Arneth Counts.

An increase in band cells and immature forms is called a "shift to the left."

2. A low leukocyte count with an increase in band cells is considered a "degenerative shift to the left."
3. A high leukocyte count with an increase in band cells, metamyelocytes, and myelocytes is considered a "regenerative shift to the left."
4. An increase in band forms indicates an infection; if the percentage is above 50, the infection is severe.
5. A continued or increased shift to the left is unfavorable, while a shift back to normal indicates a good prognosis.
6. A "shift to the right," an increase in the per cent of older cells (hypersegmentation), occurs in pernicious anemia.

E. *Toxic Degeneration.*

1. Toxic granules, vacuoles, and basophilic cytoplasm appear in neutrophils in infections.
2. Toxic granules are dark blue, fine or coarse according to the severity of the infection.
3. If the degenerative index, which is the number of cells showing toxic granules divided by the total neutrophil count, remains high, the prognosis is very grave.

F. *Normal Leukocytes.*

1. For detailed description of the cells see Table 10 and for their absolute values see Table 8.
2. *Granulocytic Series.*
   a. *Polymorphonuclear Neutrophil.*
      1) Band (nonfilament) cell (2 to 6 per cent).
      2) Segmented (filament) cell (50 to 70 per cent).
   b. *Polymorphonuclear Eosinophil* (1 to 3 per cent).

TECHN

c. *Polymorphonuclear Basophil* (0 to 1 per cent).

3. *Lymphocytes* (20 to 40 per cent).
Classify small and large lymphocytes together in the differential count.
4. *Monocytes* (2 to 8 per cent).

G. *Abnormal Leukocytes.*

1. See Table 10 for a detailed description of the cells that normally appear in the bone marrow but are abnormal when appearing in the peripheral blood. Other abnormal cells are described below.
2. In abnormal peripheral blood, the primitive cells found may vary a great deal from those found in the normal bone marrow.
3. *Granulocytic Series.*
    a. *Myeloblast* (see Table 10).
    These cells vary in size; when 8-12 microns they are called micromyeloblasts, and when 16-20 microns they are called macromyeloblasts.
    Found in myelocytic leukemias.
    b. *Promyelocyte* (see Table 10).
    Found in myelocytic leukemias.
    c. *Myelocyte* (see Table 10).
    Found in myelocytic leukemias, marked leukocytosis, and pernicious anemia.
    d. *Metamyelocyte* (see Table 10).
    Increased in same diseases as myelocytes.
    e. *Toxic Neutrophil.*
    Nucleus and chromatin–usually as in a normal neutrophil, but sometimes may be pyknotic.
    Cytoplasm—stains blue.
        Granules—basophilic, may be small and uniformly distributed or large and few in number.
        Vacuoles—may be present in the cytoplasm.
    Found in severe infections.
    f. *Döhle's Inclusion Bodies.*
        1) Occur in neutrophils.
        2) Size of micrococci or little larger.
        3) Vary in shape from pear, short rod, to coccal forms lying in pairs.
        4) Found especially in scarlet fever, sometimes in diphtheria, pneumonia, and leukemia.
4. *Lymphocytic Series.*
    a. *Lymphoblast* (see Table 10).
    It is sometimes impossible to distinguish these cells from myeloblasts. The predominance of either lymphocytes or myelocytes on the slide is generally the distinguishing point. Found in lymphocytic leukemias.
    b. *Rieder Cell.*
    Lymphoblast, myeloblast, or monoblas with a lobulated nucleus or 2 nuclei. Foun in leukemias.
    c. *Prolymphocyte* (see Table 10).
    Size—11 to 20 microns.
    Nucleus—large, round, oval, or indented usually contains nucleoli.
        Chromatin — trabeculae are not a coarse as in lymphoblast.
    Cytoplasm—not as deep blue and slightl more abundant than in the lymphoblast
        Granules—azure granules rare.
    Found in lymphocytic leukemias, infectious mononucleosis, and infectious hepatitis.
    d. *Pathological Large Lymphocyte* (15-2 microns).
        1) *Type I.*
        Nucleus — nearly fills cell, eccentric markedly lobulated.
            Chromatin — coarse, network o heavy strands.
        Cytoplasm — deep blue, vacuolated foamy, mottled or stippled appearance
            Granules — may have azure granules.
        2) *Type II.*
        Nucleus—small and rarely lobulated
            Chromatin—strands are very coars with some dense masses.
        Cytoplasm—less basophilic, fewer vacuoles, and smoother appearance than Type I.
            Granules—none.
        3) *Type III.*
        Nucleus—round or oval.
            Chromatin—fine sieve-like arrangement similar to blast cells.
        Cytoplasm—medium blue with vacuoles.
            Granules—none.
    Found in infectious mononucleosis and infectious hepatitis.
    e. *Türck's Irritation Cell* (Proplasmacyte).
    Size—12 to 20 microns.
    Nucleus—large, round, or oval, eccentrically placed, and taking a deep purplis red stain. May or may not have nucleo
        Chromatin–homogeneous.
    Cytoplasm—dense, opaque, deep blue cytoplasm with a perinuclear light area.
        Vacuoles—often present.
        Granules—none.
    Found in plasma cell leukemia, multipl myeloma, and rubella.

Note: Some hematologists believe that there is a separate plasmacytic series, while others believe the Türck's irritation cell (proplasmacyte) and plasma cell (plasmacyte) are derived from the lymphoblast. Those believing in the plasmacytic series call a cell similar to Türck's irritation cell, but having a finer chromatin structure with definite nucleoli in the nucleus and without a perinuclear light area, a plasmablast.

f. *Plasma Cell* (Plasmacyte).
   See note under Türck's irritation cell.
   Size—8 to 20 microns.
   Nucleus—eccentric and smaller than that of Türck's irritation cell.
      Chromatin—arranged in clumps like spokes of a wheel.
   Cytoplasm–similar to Türck's cell but usually not as deep a blue.
      Granules—none.
   Found in plasma cell leukemia, other leukemias, multiple myeloma, rubella, and chronic infections.

. *Monocytic Series.*
   a. *Monoblast* (see Table 10).
   b. *Promonocyte* (see Table 10).
      Found in monocytic leukemia.

. *Degenerated Forms.*
   a. These consist of bare nuclei from ruptured cells and vacuolated leukocytes.
   b. Smudges are fairly well preserved nuclei without cytoplasm, irregular in outline, stain violet, and range from 12 to 20 microns in diameter.
   c. "Basket cells" consist of a coarse network of strands of palely stained nuclear substance more than 20 microns in diameter.

. ***Megakaryocyte Series.***

Megakaryoblast.
Size—40 microns or more.
Nucleus—large and irregular, finely reticulated with distinct nucleolus.
   Chromatin—finely reticulated.
Cytoplasm—light blue and small amount.
   Granules—none.

Megakaryocyte.
Size—40 microns or more.
Nucleus—diffusely stained, indented or lobulated.
   Chromatin–arranged like the weave of a basket.
Cytoplasm—basophilic.
   Granules—distinct azurophilic.

Platelets (thrombocytes).
a. Spheric or ovoid reddish to violet bodies, 2 to 4 microns in diameter.
b. Usually appear clumped in masses of 2 to 20.
c. Only a rough estimation of an increase, decrease, or abnormal size can be made from a routine blood smear.
d. A special technique is necessary to count platelets because they clump or break up as soon as they come in contact with air.
e. See method for counting platelets on page 85.

III. Erythrocytes (Wright's Stain).

A. ***The normal erythrocyte*** (normocyte) is a biconcave disk, varying from 6.7 to 8.0 microns in diameter.

1. The central, thinner area stains paler than the periphery.
2. The cell is acidophilic and with proper staining appears orange or pink colored.

B. ***Abnormal Erythrocytes.***

1. *Variation in Size—Anisocytosis* (report 1 to 4 plus).
   a. *Microcyte*—very small erythrocyte (6.7 microns or less).
   b. *Macrocyte*—abnormally large erythrocyte (8 to 12 microns).
   c. *Megalocyte*—extremely large erythrocyte (12 to 25 microns).
   d. *Schizocytes*—fragments of erythrocytes (2 to 3 microns).

2. *Variation in Shape* (report 1 to 4 plus).
   a. *Poikilocytes* are very irregularly shaped erythrocytes. Their presence indicates blood destruction. They are not to be confused with a slight distortion which sometimes results from too much pressure while making the smear.
   b. *Spheroid cells* are thicker than normal and are found in chronic hemolytic jaundice. They hemolyze more readily than normal cells in hypotonic salt solution. This defect of the erythrocytes is hereditary.
   c. *Elliptical or oval cells* are elongated cells with rounded ends occurring in normal individuals of both the white and the colored race as an inherited trait. They do not increase in moist preparations. They are increased in anemias occurring in people having this hereditary trait.
   d. *Sickle cells* are sickle-shaped with sharp points and are best seen in a moist drop preparation. They are found exclusively in Negroes and about 8 per cent of that race inherit this trait. Sickle cells are found in anemias occurring in people with this hereditary trait.

1) *Capillary Method.*
    a) Place a tiny drop of blood on a slide, cover immediately with a cover glass, and seal with vaseline.
    b) Place in a 37°C. incubator for 18 to 24 hours.
    c) Examine with the high power objective.
    d) The erythrocytes in persons with this hereditary trait will take a sickle shape.
    e) This test is used to distinguish sickle cells from elliptical cells.
    f) In order to hasten the formation of sickle cells a rubber band may be placed around the proximal portion of the finger and allowed to remain 5 minutes before puncturing the distal end. Make a moist preparation as described above. About 70 per cent of cases harboring the trait will show sickling at the end of 1 hour; the other 30 per cent will not show sickling until later.

2) *Venous blood method.*
    a) Place 2 cc. of 10% formalin saline solution in a small test tube.

Neutral formalin	10 cc.
Distilled water	90 cc.
Sodium chloride	850 mg.

    b) Cover with a layer of mineral oil.
    c) Rinse a 5 cc. syringe with mineral oil.
    d) Place a sterile needle on the syringe and withdraw 2 cc. of blood from a vein.
    e) Leaving the needle on the syringe, immediately deliver 1 cc. of the blood below the layer of oil in the test tube.
    f) Mix well by stirring with a glass rod.
    g) Let stand 10 minutes.
    h) With a capillary pipette remove a small drop of the blood mixture, place on a slide, and cover with a cover glass.
    i) Examine for sickle cells with the high power or oil immersion objective of the microscope.
    j) Sickle cells are found in cases of sickle cell anemia by this method, but only a rare sickle cell is found in persons who have only the sickle cell trait.

e. *Target cells* are abnormally thin erythrocytes which have a tendency to buckle and present a bull's eye appearance in stained smears. They show a peripheral ring of hemoglobin separated by a clear unstained zone from a dense center. They are more resistant to hypotonic NaCl solution than normal erythrocytes.

3. *Variation in Hemoglobin Content* (report 1 to 4 plus).
    a. *Normochromia*—normal amount of hemoglobin.
    b. *Hyperchromia* — excess of hemoglobin which is probably due to increase in size of cell instead of increase in concentration of hemoglobin. Seen in macrocytic anemias.
    c. *Hypochromia*—the central pale area of the erythrocyte is larger and paler. In extreme instances the cells become mere rings (pessary forms). Seen in iron deficiency anemias.
    d. *Polychromatophilia* — cytoplasm stains from a faint bluish tinge to a rather deep blue when severe. Sometimes only a part of a cell is affected. It is a sign of youth of the cell, due to retained basophilic substance of an earlier stage. It is seen only when young cells are being quickly produced and rapidly discharged from the bone marrow and is evidence of blood regeneration.

4. *Nucleated Erythrocytes.*
    a. Report number of nucleated erythrocytes per 100 leukocytes in the differential count.
    b. *Megaloblast* (see Table 10).
    Very rare but found with rapid cell regeneration in fatal pernicious anemia, phenylhydrazine poisoning, severe anemia in infants, and acute leukemias.
    c. *Erythroblast* (see Table 10).
    Found in Cooley's anemia, in erythroblastosis fetalis, and in leukemias.
    d. *Normoblast* (see Table 10).
    Found in rapid red blood cell regeneration.

5. *Stippling.*
    a. *Basophilic*—dark blue granules ranging in size from the limit of microscopic vision up to 1 micron in diameter which are seen in the erythrocytes.
        1) Some cells exhibit polychromatophilia, because the granules may be so fine that the cell appears dusted with them.
        2) Report 1 to 4 plus, one representing a very few stippled cells on the slide and four the presence of stippled cells in every oil immersion field.
        3) For special staining see page 49.
        4) Found in cases of lead poisoning, severe anemias, leukemias, and malignant tumors.

b. *Azurophilic* — reddish-purple granules which are occasionally seen in erythrocytes.

c. *Malarial* — fine purplish-red granules. Found in erythrocytes containing malarial parasites.

6. *Reticulocytes.*
   a. Cells having dark blue filaments, skeins, wreaths, or dots when vitally stained with cresyl blue.
   b. For staining method see page 50.
   c. They are easily stained if the staining is done while the cell is still alive, but do not stain in the usual dried smear.
   d. They are usually larger and paler than normal adult erythrocytes.
   e. *Interpretation.*
      1) They are immature erythrocytes and represent a stage of development between the normoblast and adult cell.
      2) Normal blood has 0.5 to 1.5 per cent.
      3) A decrease in reticulocytes is characteristic of aplastic anemia.
      4) They are increased in chronic hemolytic jaundice, sickle cell anemia, Cooley's anemia, myelocytic leukemia, and metastatic malignant disease of the bone marrow.
      5) A marked increase occurs in anemias of infections such as malaria, typhoid fever, brucellosis, and pneumonia, also in intoxications such as lead or benzol poisoning.
      6) A temporary marked increase occurs following acute hemorrhage and in spontaneous remissions of pernicious anemia.
      7) Often a mild increase occurs after a transfusion.
      8) A transient sharp rise in the reticulocyte count begins 2 to 6 days after the institution of specifically effective therapy (liver, iron, folic acid, or vitamin C) and lasts 3 to 7 days. The height of the reticulocyte peak depends not only on the adequacy of the dose and the activity of the therapeutic agent but also on the initial level of the erythrocyte count. See Table 9.
      9) There is a marked increase after splenectomy.

. *Howell-Jolly Bodies.*

a. They are retained spherical nuclear particles about 1 micron in diameter and stain deep blue.

b. There may be one or more bodies in a cell and are usually in the periphery of the cell.

TABLE 9. RETICULOCYTE RESPONSE IN TREATMENT
(after Haden)

Initial R.B.C. (millions)	Average reticulocyte peak in per cent	
	With adequate oral treatment	With adequate intramuscular treatment
0.5 - 1.0	55	56.8
1.0 - 1.5	35	40.0
1.5 - 2.0	22	28.0
2.0 - 2.5	14	28.0
2.5 - 3.0	8	19.0
3.0 - 3.5	3	6.4
over 3.5	1	1.8

8. *Cabot Rings.*
   a. These remnants of the nuclear membrane are left when the nuclear chromatin is dissolved from within them.
   b. They are seen as rings or figure-eight-shaped structures which stain red or reddish purple.
   c. They are a sign of abnormal maturation of the nucleus.
   d. Found in severe anemias and especially numerous after splenectomy.

Examination of Bone Marrow

I. Sternal Puncture.

A. *Obtaining the Specimen.*

1. Aseptic precautions are used throughout the procedure.
2. Paint an area of skin over the middle of the sternum with tincture of iodine and then wash with 70% alcohol.
3. Infiltrate with 1% novocaine an area of skin just below the junction of the body of the bone with the second rib.
4. Continue to inject straight down to the sternum so the infiltration includes the periosteum over the bone.
5. After anesthesia is complete, a 10 gauge short needle (sternal puncture needle) with the stylet in place is inserted at an angle of about 45 degrees until it reaches the periosteum.
6. The pressure on the needle is continued until it enters the bone marrow.
7. The stylet is removed and a 10 cc. syringe is connected to the needle.
8. Suction is made with the syringe and about 0.3 cc. of grayish bone marrow with blood from the sinusoids is aspirated.

B. *Making Smears.*

1. Place small drops of the marrow on glass slides and smear the same as for blood smears.
2. Stain with Wright's stain, leaving the stain on 2 minutes; then add buffer and stain for 10 minutes. The smears are more difficult to stain because of the fat present.

TABLE 10. MORPHOLOGY OF BLOOD CELLS (WRIGHT STAIN)

Type of Cell	Size‡ in microns	Nucleus						Cytoplasm			
		Position in Cell	Shape	Staining‡‡ Quality	Chromatin	Nuclear Membrane	Nucleoli	Relative Amount	Color‡‡	Perinuclear Zone	Granules
1. GRANULOCYTES a. Myeloblast*	8 - 20	Eccentric	Round or oval	Light reddish purple	Finely reticulated, almost homogeneous	Indistinct	3 - 5 distinct	Narrow rim	Deep blue	None	None
b. Promyelocyte	10 - 20	Eccentric or central	Round or oval	Reddish purple	Finely reticulated, net form	Present	0 - 3	Moderate	Less deeply blue	Sometimes present	Few scattered coarse deeply azurophilic or few small lilac near nucleus
c. Myelocyte	10 - 18	Eccentric or central	Oval or sl. indented	Reddish purple	Reticulated, becoming more coarse and compact	Indistinct	Usually none	Moderate	Bluish pink	None	May be fine or coarse neutrophilic, eosinophilic, or basophilic
d. Metamyelocyte	10 - 16	Eccentric or central	Kidney to wide band	Light purplish blue	Fine strands, becoming more compact	Present	None	Large	Pink	None	Neutrophilic, eosinophilic, or basophilic
e. Band or nonfilament	10 - 15	Eccentric or central	Curved narrow band or convoluted	Deep purplish blue	Coarse strands	Present	None	Large	Pink	None	Neutrophilic, eosinophilic, or basophilic
f. Polymorphonuclear neutrophil	10 - 15	Eccentric or central	2 - 5 or more lobes	Deep purplish blue	Coarse interlacing bands	Present	None	Large	Faint pink	None	Violet, uniformly fine, diffusely scattered
g. Polymorphonuclear eosinophil	10 - 15	Eccentric or central	2 - 3 lobes	Pale purplish blue	Coarse	Present	None	Large	Bluish pink	None	Numerous, uniformly large, bright red, **sometimes bronze**
h. Polymorphonuclear basophil	8 - 14	Eccentric or central	Covered with granules	Pale purplish blue	Coarse	Present	None	Moderate	Faint pink	None	Few to many coarse bluish black, sometimes varying in size
2. LYMPHOCYTES** a. Lymphoblast*	8 - 20	Eccentric or central	Round, oval or indented	Light reddish purple	Moderately coarse, "stippled"	Fairly dense	1 -3	Small to moderate	Deep blue to sky blue	Present	None
b. Prolymphocyte	16 - 20	Eccentric	Round, oval or sl. indented	Purplish blue	Beginning to clump	Dense	0 - 3	Large	Deep blue to sky blue	Sometimes present	May or may not have a few azure
c. Lymphocyte ***	7 - 15	Eccentric	Round, oval or sl. indented	Deep purplish blue	Heavy masses and clumps	Dense	None	Small	Sky blue to deep blue	Usually not	May or may not have a few azure
3. MONOCYTES a. Monoblast*	15 - 20	Eccentric	Round or oval	Purplish red	String-like	Very fine	2 - 5 very small	Small sometimes having pseudopodia	Deep blue	None	None
b. Promonocyte	12 - 20	Eccentric	Indented or oval	Pale reddish purple	Fine reticulum	Very fine	None	Moderate often with pseudopodia	Cloudy blue	None	May or may not have a few fine lilac
c. Monocyte	10 - 22	Eccentric	Indented or convoluted	Reddish purple	Loosely meshed interlacing strands	Faint	None	Large often with pseudopodia	Cloudy blue	None	Dusted with fine lilac
4. ERYTHROCYTES a. Megaloblast*	10 - 20	Central	Round or oval	Purplish red	Delicate network	Distinct	Usually none	Small (¼ of cell)	Deep blue to gray blue	Usually present	None
b. Erythroblast	8 - 12	Eccentric or central	Round or oval	Deep purplish blue	Coarse, in clumps	Distinct	None	Moderate (½ of cell)	Blue to pink	None	None
c. Normoblast	7 - 9	Eccentric or central	Round, irregular, lobulated, or fragmented	Deep purplish blue	Irregular, compact or pyknotic	Distinct	None	Moderate (less than ½ of cell)	Blue to pink	None	None

‡ The size of cell varies with the thickness of the smear.

‡‡ The color varies with the staining time and the thickness of the smear.

* ... to distinguish between early myeloblasts, lymphoblasts, monoblasts and megaloblasts; they are best called blast or stem cells.

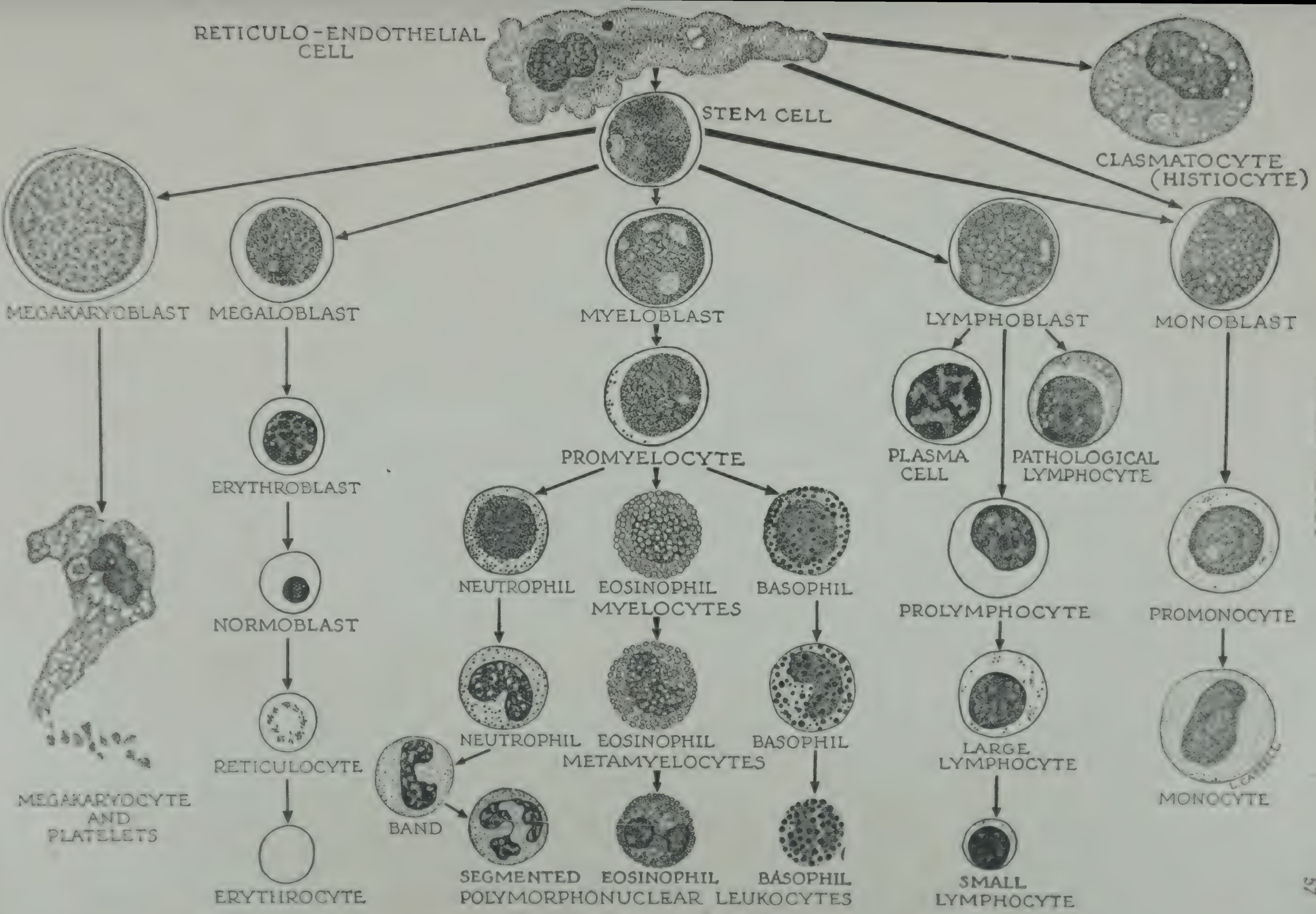
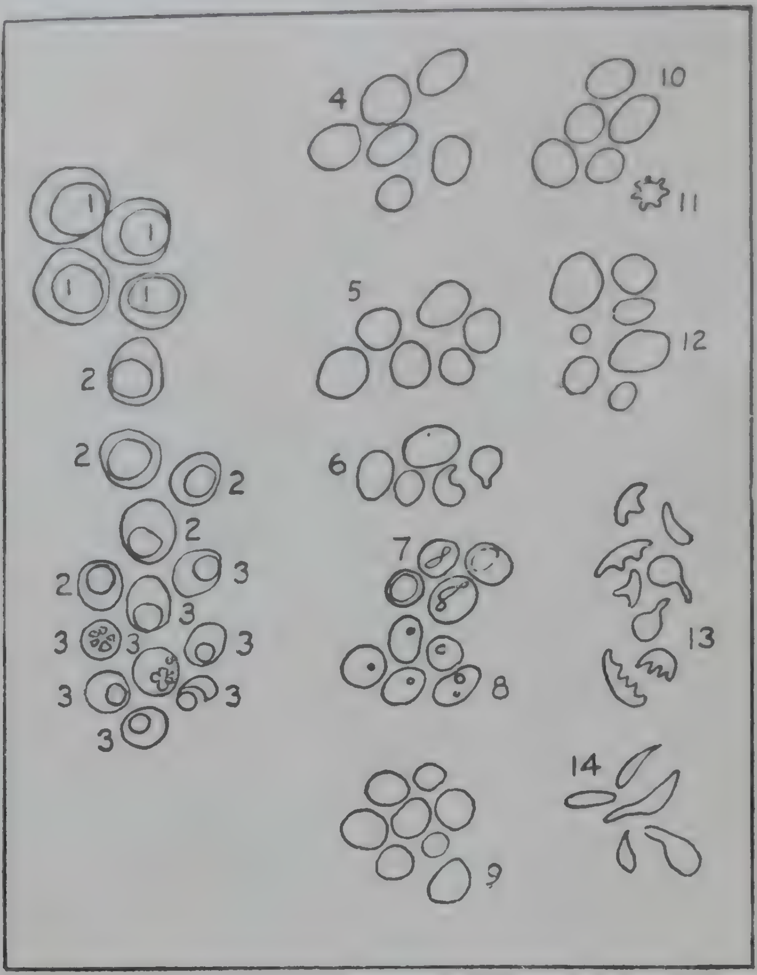

RETICULO-ENDOTHELIAL CELL
STEM CELL
CLASMATOCYTE (HISTIOCYTE)
MEGAKARYOBLAST
MEGALOBLAST
MYELOBLAST
LYMPHOBLAST
MONOBLAST
PLASMA CELL
PATHOLOGICAL LYMPHOCYTE
PROMYELOCYTE
ERYTHROBLAST
NEUTROPHIL
EOSINOPHIL
BASOPHIL
MYELOCYTES
PROLYMPHOCYTE
PROMONOCYTE
NORMOBLAST
NEUTROPHIL
EOSINOPHIL
BASOPHIL
METAMYELOCYTES
RETICULOCYTE
LARGE LYMPHOCYTE
MEGAKARYOCYTE AND PLATELETS
BAND
MONOCYTE
SEGMENTED
EOSINOPHIL
BASOPHIL
POLYMORPHONUCLEAR LEUKOCYTES
ERYTHROCYTE
SMALL LYMPHOCYTE
L. CAHILL

FIG. 9. DEVELOPMENT OF BLOOD CELLS.



KEY TO PLATE 1

Maturation in Erythrocyte Series

1. *Megaloblasts:* Nucleus is broken up in appearance and dark purple in staining reaction. Cytoplasm is deeply basophilic, irregular in staining, with clear zones of lighter color toward the center.

2. *Erythroblasts:* Nucleus is more condensed but still broken up in appearance and darkly staining, with coarser clumps of chromatin. Cytoplasm is less basophilic, though varying amounts of bluish color are still present; at the periphery, definite reddish hemoglobin color is apparent through the basophilia.

3. *Normoblasts:* Nucleus is dense and pyknotic, in many instances fragmented and occasionally partly extruded. Cytoplasm is the usual reddish color of hemoglobin, the basophilia having almost completely disappeared.

4. *Polychromatophilic erythrocytes or polychromatocytes:* Young erythrocytes, still retaining some of the basophilic staining reaction; with the reddish hemoglobin background, a multicolored appearance results and therefore the name.

5. *Punctuate basophilic stippling,* with black to bluish granules in the cells. This is usually seen in severe anemia and in toxic disturbances.

6. *Hypochromic erythrocytes:* The concentration of the hemoglobin is deficient, usually indicating iron lack or interference with hemoglobin synthesis. Cells often have only a ring of hemoglobin at the periphery, and usually are small.

7. *Cabot rings:* Remnants of nuclear membrane, appearing as circles or figures-of-eight in the cells.

8. *Howell-Jolly bodies:* Nuclear remains of varying size, staining dark blue or black.

9. *Reticulocytes:* Young forms of erythrocytes, staining characteristically with a vital dye, such as cresyl blue, before counterstaining with Wright's. The reticulum, a fine network of blue or purple threads, may be concentrated in the center of the cell or spread out diffusely; occasionally it will appear almost punctuate in character.

10. *Normal mature erythrocytes or normocytes:* Cells are about 7 microns in diameter and contain enough hemoglobin to give the whole cell a pinkish-red stain except at the biconcave center where very little stain is evident.

11. *Pyknotic erythrocyte:* An artifact often produced by faulty fixation or staining of blood films.

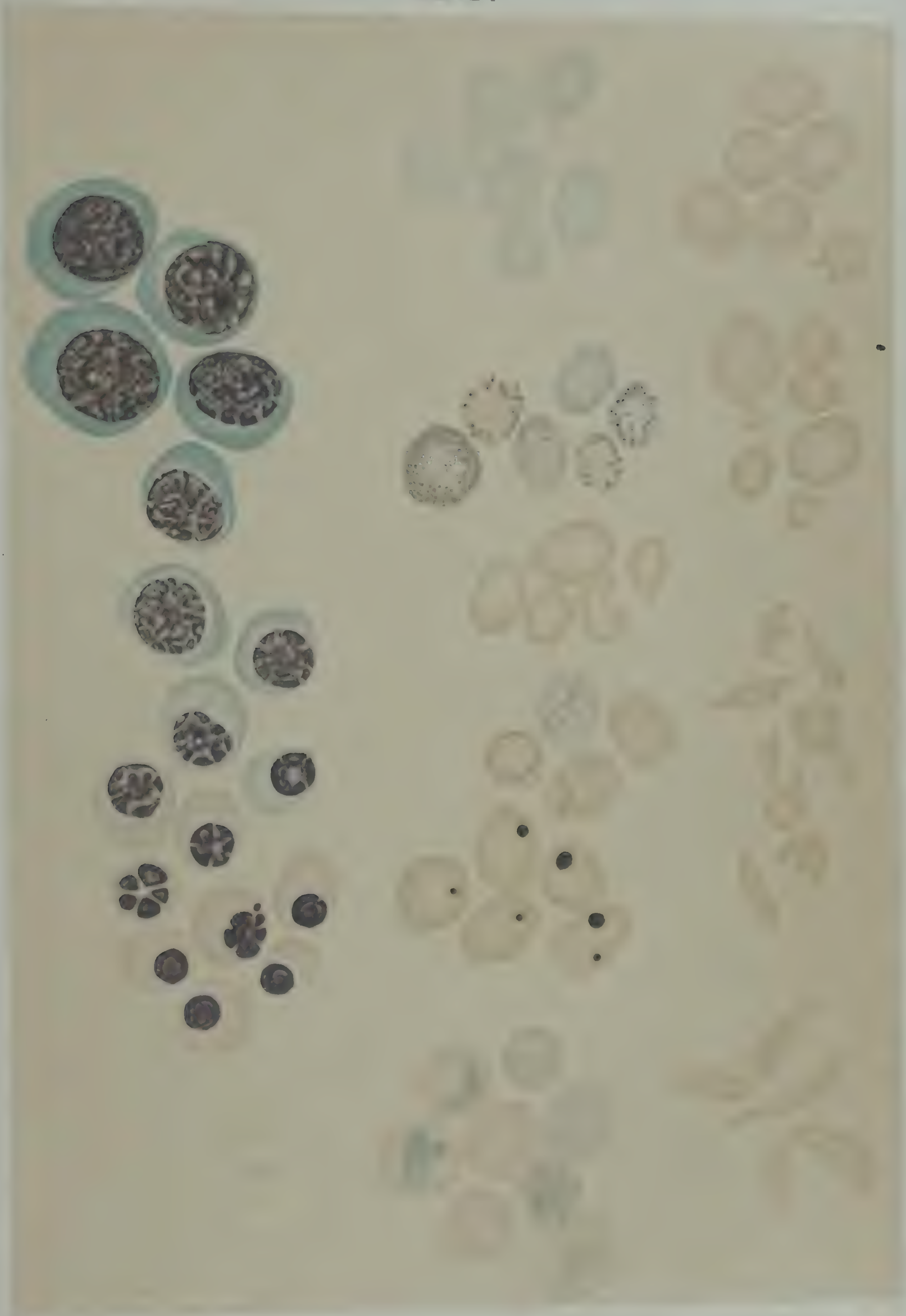
12. *Macrocytes* and *microcytes:* Cells larger and smaller, respectively, than normal.

13. *Poikilocytes:* Cells of abnormal shape, most commonly tailed forms.

14. *Sickle cells:* Cells pulled out of normal shape into sickled form. This trait is found almost exclusively in Negro blood; it is best produced by depriving susceptible fresh cells of oxygen or exposing them to carbon dioxide.

(Plates I-V from Blackfan, Diamond, and Leister's "Atlas of the Blood in Children," courtesy of the Commonwealth Fund, New York City.)

PLATE 1



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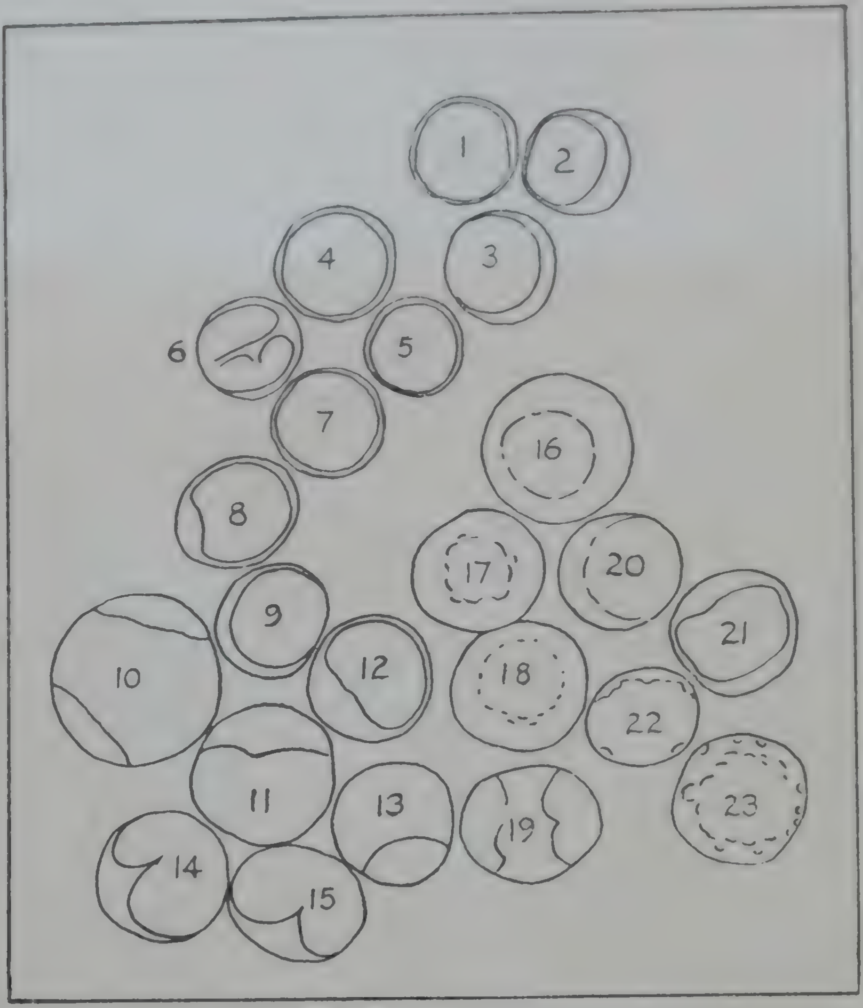


KEY TO PLATE 2

Maturation in Myeloid Series

1-6. *Myeloblasts:* Nucleus is large with one or more nucleoli; an occasional nucleus is in process of division. Cyt plasm is scant and intensely basophilic.

7, 8. *Myelocytes type "A," or promyelocytes:* Nucleus is more condensed. Cytoplasm has beginning nonspecif dark granulation, often edging over the nucleus.

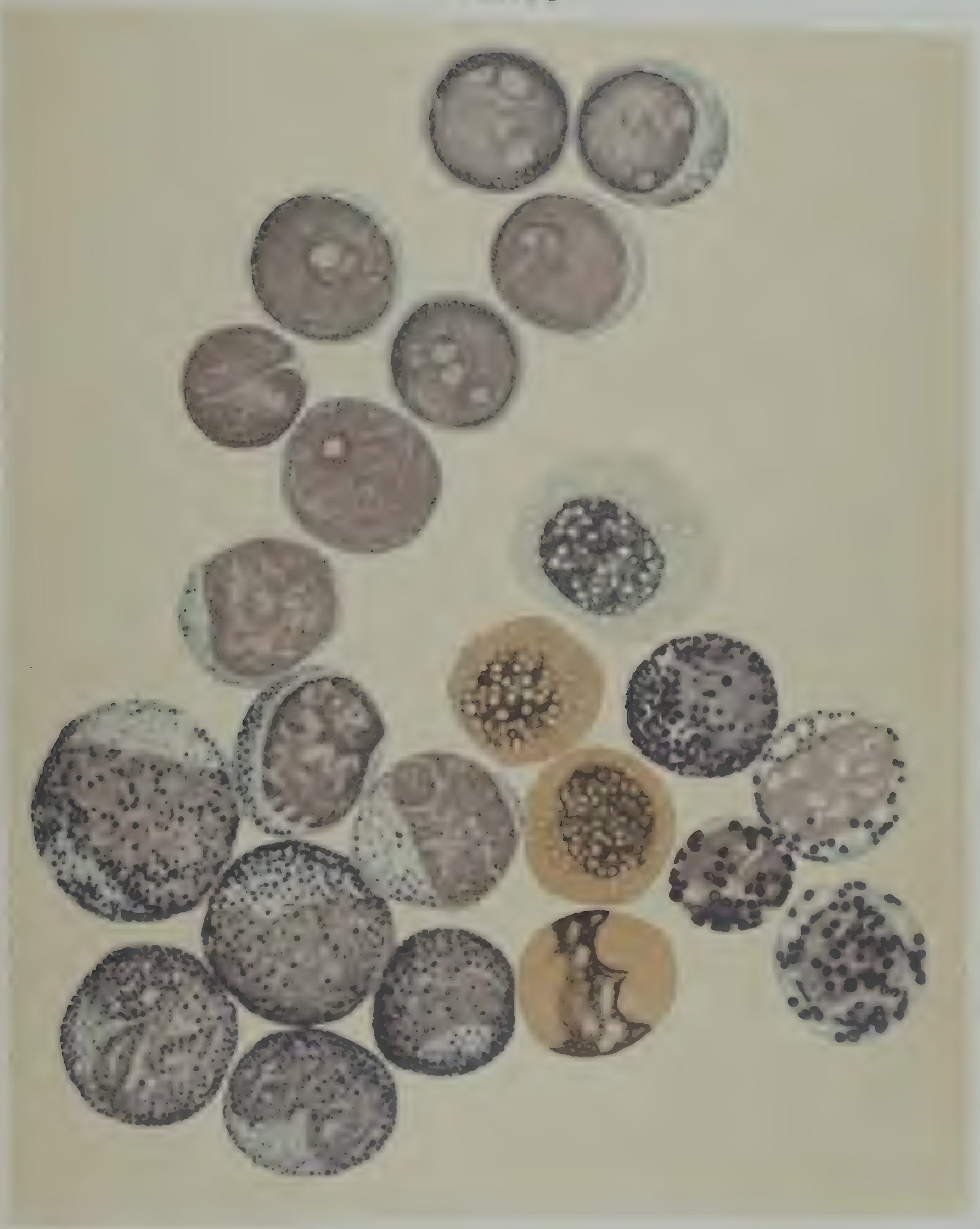
9-13. *Myelocytes type "B,"* showing increasing maturity. Nucleus is smoother, more condensed, with no nucleol Cytoplasm is more abundant, with less basophilia; granulation is still dark and nonspecific, often partly coverir the nucleus.

14, 15. *Myelocytes type "C," metamyelocytes,* or *late myelocytes:* Nucleus is often indented. Cytoplasm is fairl light blue in staining, dark, coarse granulation being replaced by fine neutrophilic granulation.

16-19. *Eosinophilic myelocytes:* Cytoplasm contains large granules of eosinophilic type, filling the cell and ofte covering and obscuring the nucleus, which is generally round and light staining.

20-23. *Basophilic myelocytes:* Nucleus is undifferentiated, light staining. Cytoplasm is filled with large, heavy, dar blue to black granules, often partly covering the nucleus.

PLATE 2



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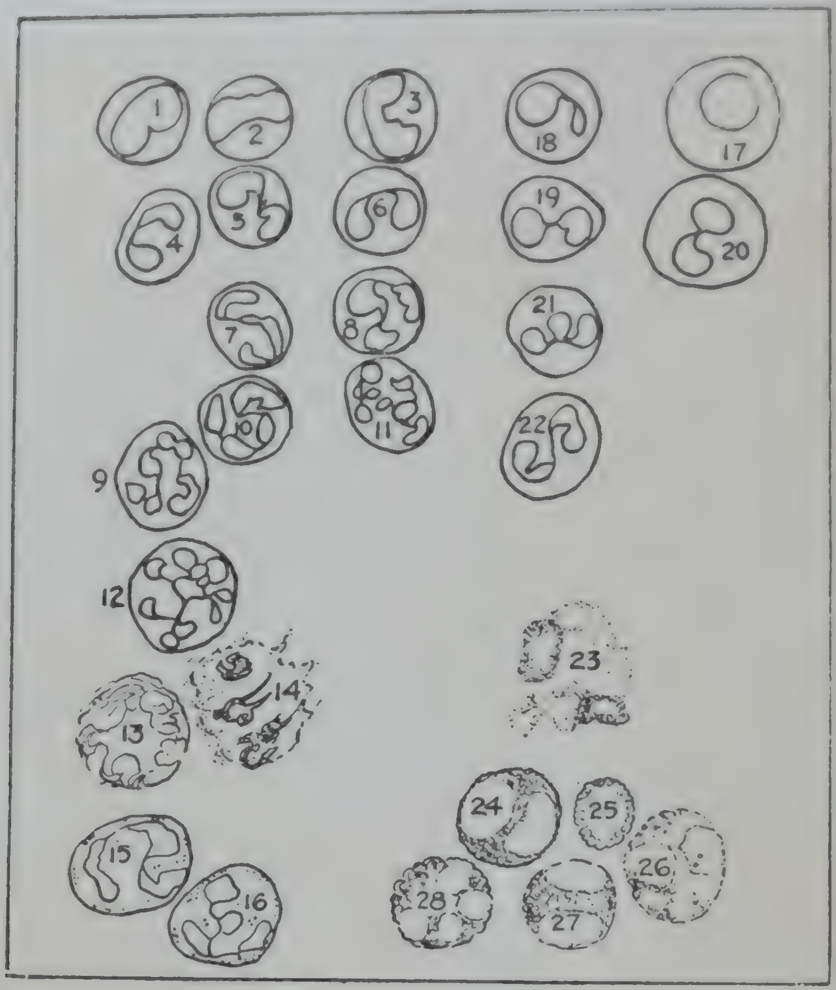


KEY TO PLATE 3

Maturation in Myeloid Series—(continued)

1-3. *Young neutrophils;* also called *early, band-form,* or *nonsegmented neutrophils.* Nucleus is condensed and beginning to lobulate. Cytoplasm contains full complement of neutrophilic granules.

4-6. *Adult neutrophils:* Nucleus has two lobes. Cytoplasm is packed with granules.

7-8. *Adult neutrophils:* Nucleus has three lobes.

9, 10. *Adult neutrophils:* Nucleus has four or more lobes.

11, 12. *Aging neutrophils:* Nucleus has multiple lobulation. Cytoplasm contains sparser granules and some vacuoles.

13, 14. *Degenerated neutrophils, artifacts:* Old and fragile cells disrupted in smearing process.

15, 16. *Adult neutrophils:* Cytoplasm contains larger but fewer granules which stain heavily—so-called toxic granules—and some vacuoles. This type of cell is common in severe infections and intoxications.

17. *Young eosinophil:* Nucleus is not lobulated as yet. Cytoplasm is packed with large eosinophilic granules.

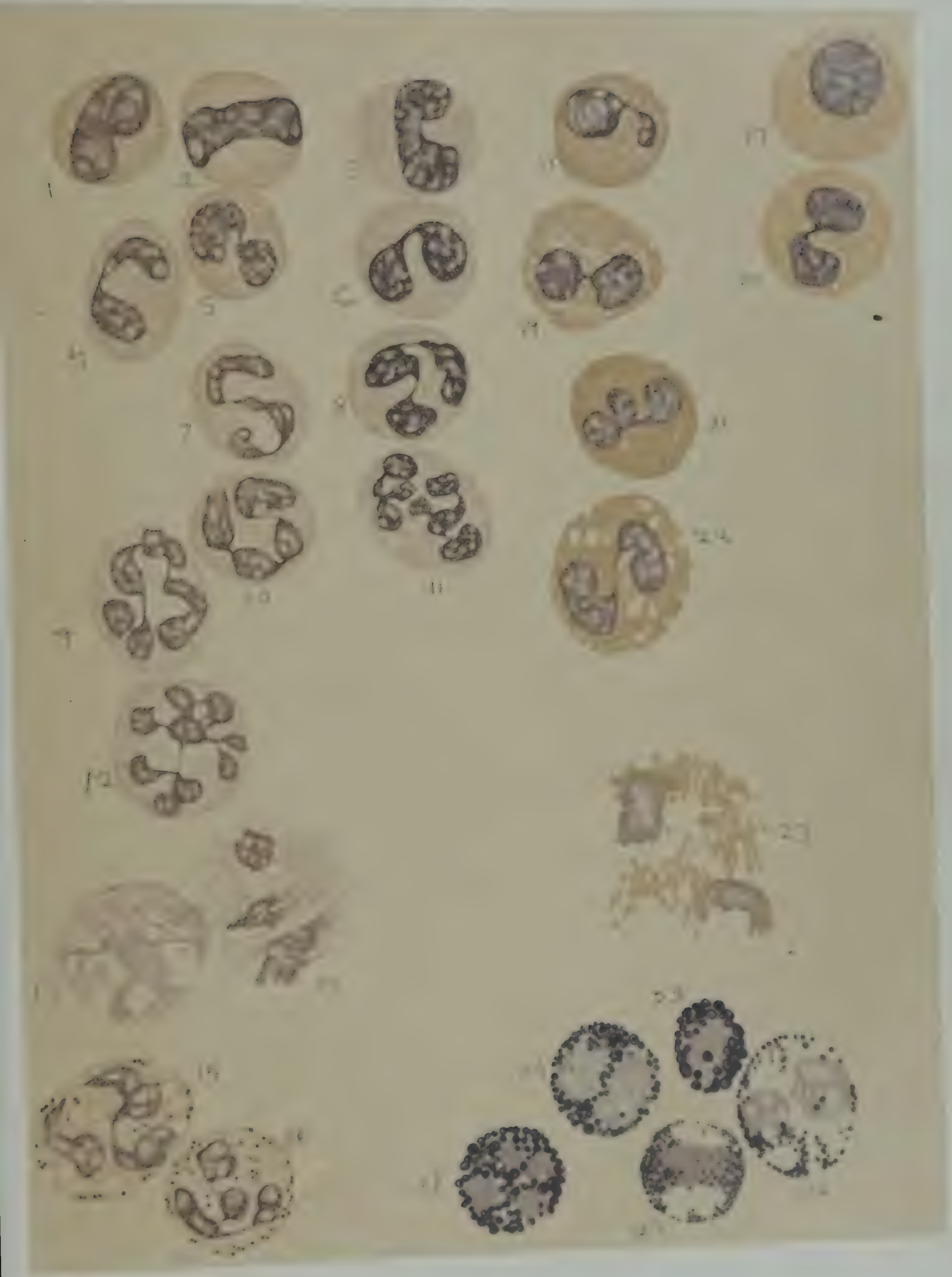
18-21. *Adult eosinophils:* Nucleus usually has two or three lobes. Cytoplasm is filled with typical granules.

22. *Aging eosinophil:* Vacuolization in cytoplasm is beginning.

23. *Aging eosinophil:* Old and fragile cell disrupted in smearing process.

24-26. *Basophils:* Nucleus usually has two or three lobes. Cytoplasm is generally filled with very large blue-black granules.

PLATE 3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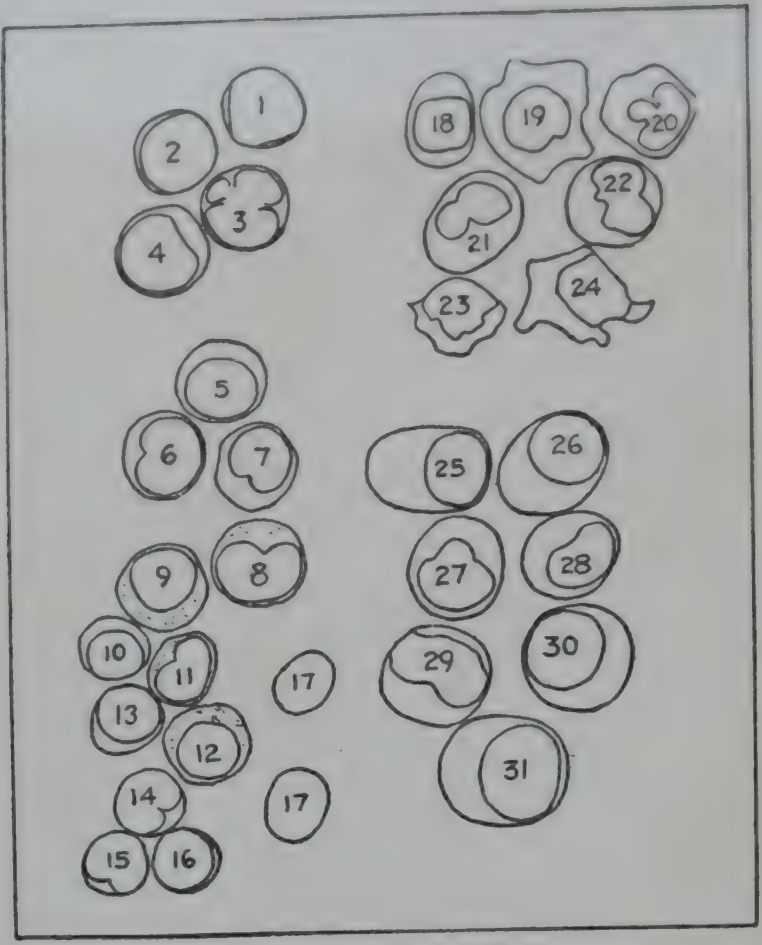


KEY TO PLATE 4

Maturation in Lymphoid Series

1-4. *Lymphoblasts:* Nucleus is large, containing one or more nucleoli; an occasional nucleus is in process of division, as in 3. Cytoplasm is scant and basophilic.

5-7. *Large* or *young lymphocytes:* Nucleus is somewhat condensed, with tendency to spoke-like arrangement o chromatin. Cytoplasm is fairly clear blue.

8, 9. *Large lymphocytes:* Nucleus is condensed. Cytoplasm is fairly abundant and is clear light blue, containin several to many large reddish granules toward periphery of cell.

10-12. *Medium lymphocytes:* Nucleus is condensed. Cytoplasm is moderate in amount, occasionally containin reddish granules.

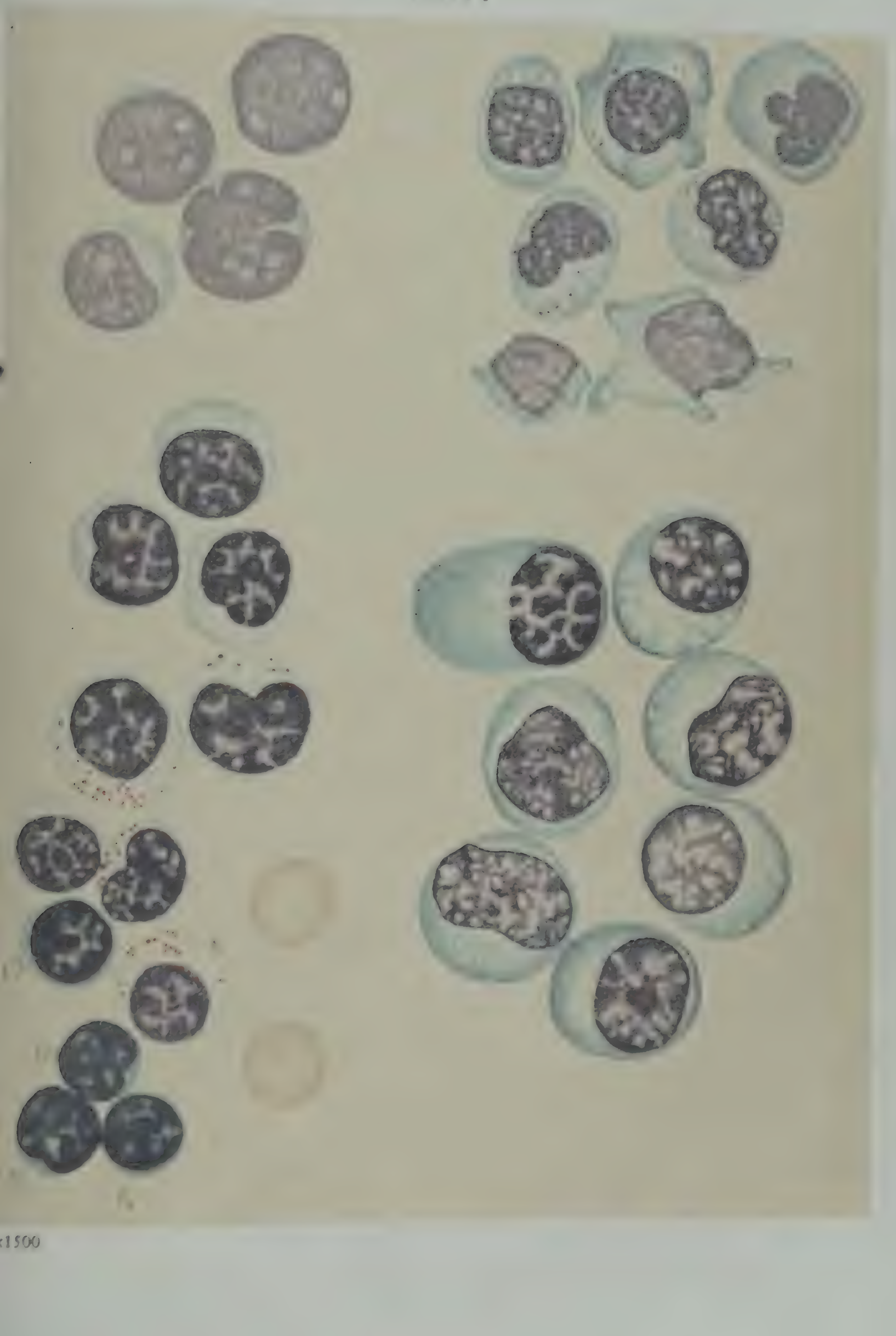
13-16. *Small* or *adult lymphocytes:* Nucleus is condensed. Cytoplasm is scanty and clear blue.

17. *Erythrocytes,* for comparison of size.

18-24. *Large lymphocytes* of the type seen most frequently in infectious mononucleosis. Nucleus is dense wit some fenestration of chromatin, and is often eccentrically placed. Cytoplasm is abundant, with irregular edge whic molds itself around adjacent cells; it is a clear light blue, denser at margin, and may contain some reddish granule

25-31. *Lymphocytes* of plasma cell type: Nucleus is eccentrically placed, darkly staining with dense chromati clumps often in cart-wheel arrangement. Cytoplasm is abundant, dark blue with clear area near nucleus, and stain more densely at periphery; it often has foamy appearance.

PLATE 4



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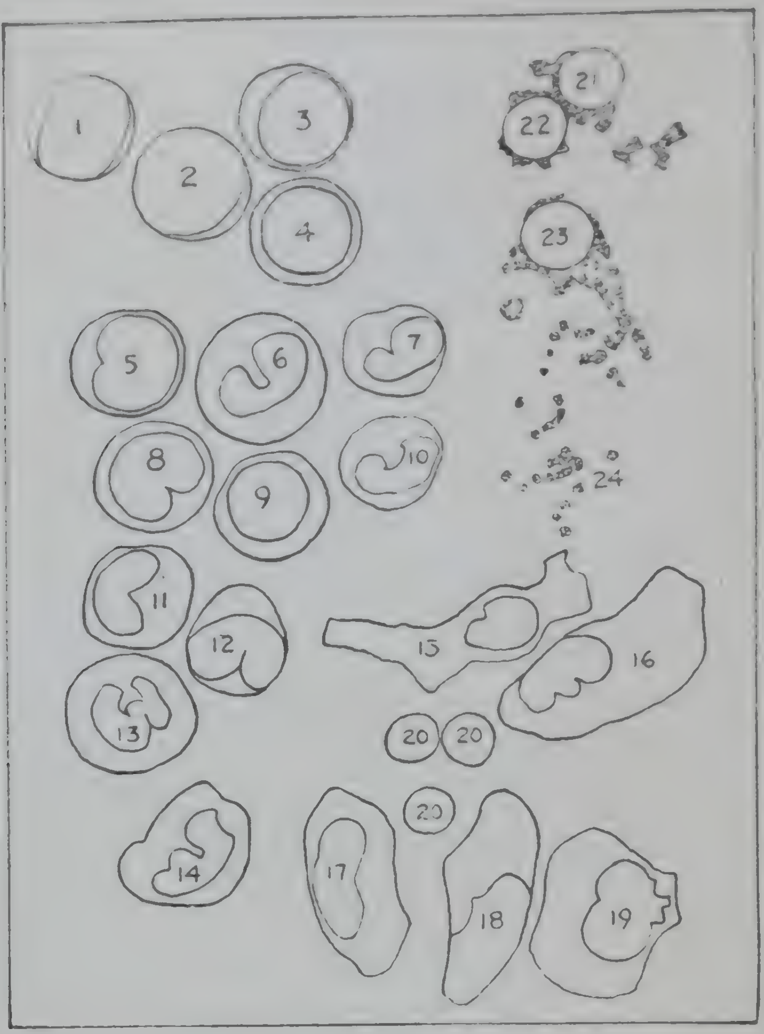


KEY TO PLATE 5

Maturation in Monocyte and Thrombocyte Series

1-4. *Monoblasts:* Large cells with relatively small amount of cytoplasm showing moderate basophilia. Nucleus large and loose in structure, but stains fairly evenly; one or more nucleoli.

5-14. *Monocytes:* Nucleus has fine chromatin network, and is often horseshoe or kidney shaped. Cytoplasm abundant and stains a light gray-blue; it contains fine reddish-blue granules, mostly toward periphery; occasiona granules are darker and more abundant, as in 7, 9, and 10; often there is a clear zone in the cytoplasm at the "h or indentation of the nucleus. Occasionally there are vacuoles, evidence of active phagocytosis, at the periphe of the cytoplasm, as in 14.

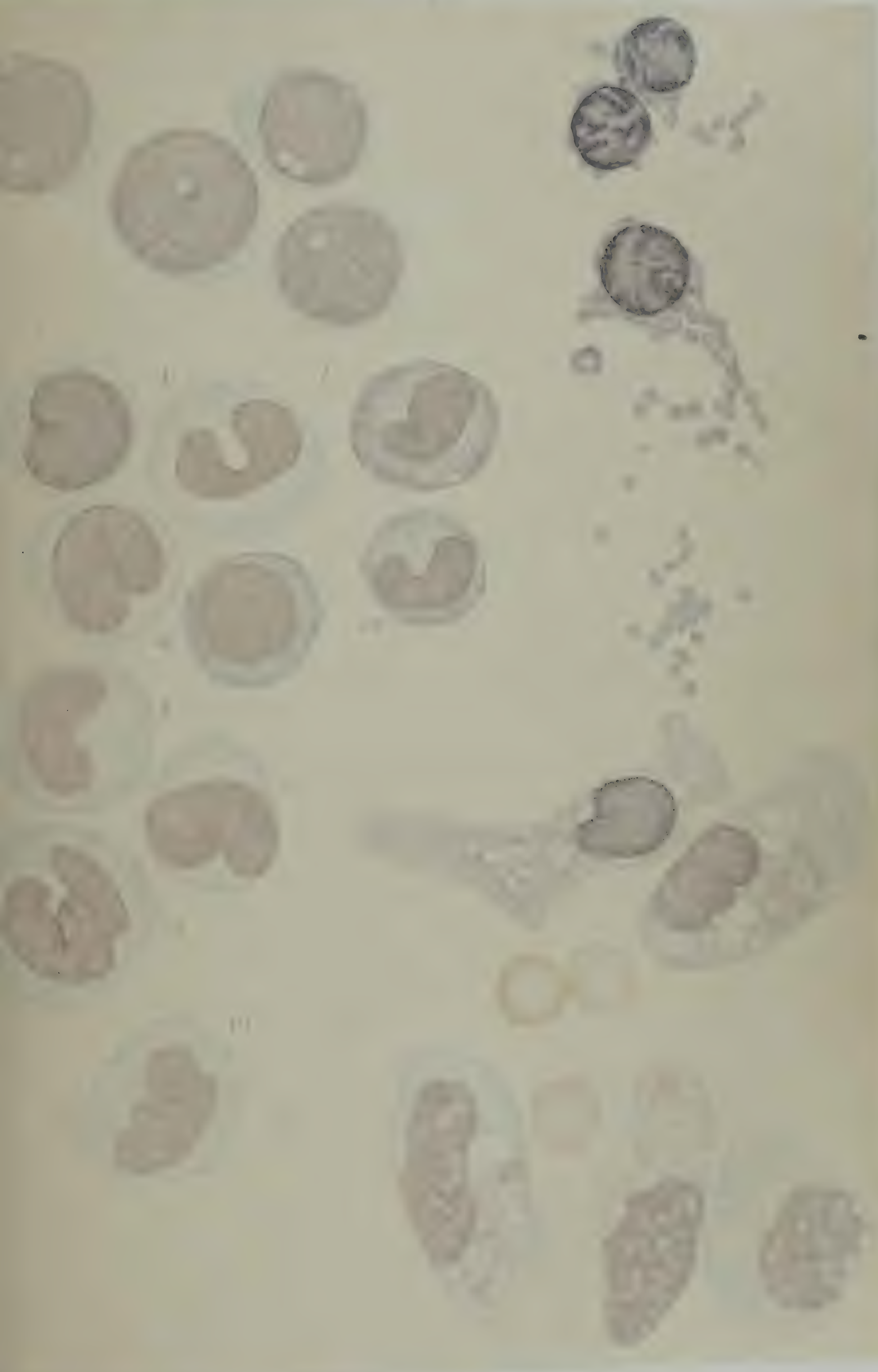
15-19. *Mononuclear phagocytes, endothelial phagocytes,* or *clasmatocytes,* the largest cells ordinarily found in t peripheral blood. Nucleus is eccentrically placed. Cytoplasm is finely granulated and contains many vacuoles all sizes, often filled with ingested material; it stains light blue.

20. *Erythrocytes,* for comparison of size.

21-23. *Megakaryocytes:* These cells are very rarely found in the peripheral blood. Nucleus is dense, often jel like, homogeneous appearance. Cytoplasm is light blue with dark purple masses of granules and no visible cellu membrane; it often trails in long pseudopods, as in 23, and masses of platelets break off and are scattered as individ platelets.

24. *Platelets* or *thrombocytes.*

PLATE 5



II. Differential Cell Count in Bone Marrow.

A. *Normal Range.*

	Per cent
Myeloblasts	0 to 5.0
Promyelocytes	1 to 8.0
Myelocytes (neutrophils)	5 to 20.0
Myelocytes (eosinophils)	0 to 3.0
Myelocytes (basophils)	0 to 0.5
Metamyelocytes (neutrophils)	5 to 10.0
Metamyelocytes (eosinophils)	0 to 2.0
Metamyelocytes (basophils)	0 to 0.3
Band (neutrophils)	15 to 35.0
Band (eosinophils)	0 to 2.0
Band (basophils)	0 to 0.6
Segmented (neutrophils)	7 to 30.0
Segmented (eosinophils)	0 to 4.0
Segmented (basophils)	0 to 0.7
Lymphocytes	4 to 16.0
Monocytes	0 to 5.0
Megaloblasts	0 to 2.0
Erythroblasts	1 to 12.0
Normoblasts	5 to 25.0
Megakaryocytes	0 to 2.0
Plasma cells	0 to 1.0
Reticulocytes	1 to 5.0

The normal myeloid-erythroid ratio in the adult ranges from 4 or 5 to 1.

B. *Pathological Findings.*

1. In the leukemias there is a marked predominance of immature leukocytes (granulocytes, lymphocytes, or monocytes) with a corresponding decrease in the erythrocyte series.
2. In agranulocytosis there is a maturation arrest of the granular cells at the myelocyte stage.
3. In infectious mononucleosis there is a myeloid hyperplasia and immaturity without any of the typical pathological lymphocytes found in the peripheral blood.
4. In pernicious anemia, in pernicious anemia of pregnancy, and in the anemias of extreme malnutrition and of sprue there is an increase of megaloblasts.
5. In hemorrhagic, hemolytic, iron deficiency, and sickle cell anemia there is an increase of erythroblasts and normoblasts.
6. In Cooley's anemia there is a hyperplasia with the erythroblasts predominating, although there is also an increase in myeloid and megakaryocyte formation.
7. In some cases of aplastic anemia, there is a quantitative decrease of all cells of the marrow with the differential count remaining essentially unchanged, while in others there is a normal or increased number of cells; the latter type of marrow reveals a good prognosis.
8. In active polycythemia there is a hyperplasia especially of the erythrocytic elements, but also of the granulocytes and megakary cytes.
9. In primary thrombocytopenic purpura the is an increase in megakaryocytes.
10. In Gaucher's disease typical Gaucher ce may be found on careful search. They a large, pale, round or polyhedral, single multinucleated cells with parallel wavy fibr in the cytoplasm.

Blood Parasites

I. Malaria.

A. *Identification of Species* (see Table 11).

1. It is best to examine blood taken during th period 10 to 12 hours after the chill to sev eral hours before the chill. See Fig. 10.
2. *Thin Smears.*
   a. Regular blood smears are made and staine longer than the regular staining time wit Wright's stain.
   b. Thin smears are better for studying th morphology of the parasite.
   c. Blood platelets lying on erythrocytes mu not be mistaken for malarial parasites.
3. *Thick Smears* (for preparation see B).
   a. A relatively large quantity of blood placed in a small area on a slide and staine with Giemsa's stain, so that the hemoglobi is dissolved from the erythrocytes makin it sufficiently transparent for examinatio by transmitted light.
   b. The organisms stain the same as in thi films but do not appear in erythrocyte only parasites, leukocytes, and platele are stained.

B. *Preparation and Staining of Thick Smears.*

1. Use new slides which have been thoroughl cleaned and are free from grease, dust, aci or alkali.
2. Cleanse the skin of the finger or lobe of th ear with gauze soaked in alcohol.
3. Rub dry with sterile gauze.
4. Prick the skin deep enough to allow th blood to well up in a large drop under gentl pressure.
5. Touch the surface of the slide to the cres of the large drop of blood and, withou losing contact with the drop of blood o touching the skin, move the slide in narro circles in the blood until a smear about th size of a dime is made.
6. Ordinary printing can just be read thro the wet center of a smear of proper thic ness.
7. Lay the slide flat to dry and cover with th

top of a Petri dish to protect from dust and insects.

Let dry 8 to 12 hours at room temperature or 30 minutes in a 37°C. incubator.

Giemsa's Stain.

a. Use buffered distilled water having a pH of 7.0 to 7.2 for diluting stain and washing smear.

M/15 Na_2HPO_4*	3.3 cc.
M/15 NaH_2PO_4*	1.7 cc.
Distilled water	45.0 cc.

*See page 259.

This buffered water must be made fresh each week and should give a greenish-blue color when a drop of bromthymol blue indicator is added to a few cc. of it.

b. Add 0.3 cc. of stock Giemsa's solution to 15 cc. of buffered distilled water.
c. Cover smear with stain and let stand for 45 minutes.
d. Pour off stain; add buffered distilled water and let stand 3 to 5 minutes.
e. Pour off water and let dry by standing slide on end.
f. The cytoplasm of the malarial organism stains blue and the chromatin a purplish red.
g. The background of the smear is a mottled pale gray to blue gray.

Field's Stain.

a. *Solution A.*
   1) Dissolve 1.3 gm. of medicinal methylene blue and 5 gm. of anhydrous Na_2HPO_4 in 50 cc. of distilled water.
   2) Bring to a boil and then evaporate on a water bath almost to dryness.
   3) Add 6.25 gm. of anhydrous KH_2PO_4 and 500 cc. of distilled water; stir until the stain is completely dissolved.
   4) Set aside for 24 hours and filter before using. Solution keeps well.
   5) The boiling and evaporating process may be eliminated by using the following formula:

Unna's polychrome methylene blue	5.00 gm.
Na_2HPO_4 (anhydrous)	5.00 gm.
KH_2PO_4 (anhydrous)	6.25 gm.
Distilled water	500.00 cc.

      a) Dissolve the phosphate salts in the water and add the dye.
      b) It is ready for immediate use after filtering.

b. *Solution B.*

Eosin or erythrosin	1.00 gm.
Na_2HPO_4 (anhydrous)	5.00 gm.
KH_2PO_4 (anhydrous)	6.25 gm.
Distilled water	500.00 cc.

   1) Dissolve the phosphate salts in the water and then add the dye.
   2) Filter before using. Solution keeps well.

c. The thick smear may be stained as soon as it appears dry and does not need to be dried for a long period of time.
d. Dip smear into Solution A for 1 second.
e. Rinse by waving gently in clean water until the stain ceases to flow from the film and the glass of the slide is free from stain (a few seconds).
f. Dip into Solution B for 1 second.
g. Rinse by waving gently for 2 or 3 seconds in clean water.
h. Place vertically against a rack to drain and dry.
i. The malarial organisms are better preserved by this staining method; the cytoplasm stains blue and the chromatin stains dark purplish red.
j. The background of the smear is a creamy-yellow color, sometimes mottled with pale blue.

C. *Life History of Parasite.*

1. *Asexual Cycle in Man* (Schizogony).
   a. *Trophozoite Stage.*
      1) *Hyaline body*—formed when a merozoite or sporozoite enters an erythrocyte.
      2) *Signet ring form*—a ring of cytoplasm surrounds the hyaline body and contains a dot of chromatin.
      3) *Amoeboid form*—the ring form enlarges, becomes actively motile, and pigment granules appear.
   b. *Schizont Stage.*
      1) *Presegmented forms*—the amoeboid form becomes compact with scalloped or irregularly notched edges. The chromatin divides into a number of masses and the pigment may or may not be clumped.
      2) *Segmented forms*—a definite number of spores are formed (merozoites) and each contains a small chromatin mass, while the pigment between the merozoites is arranged in masses near the center.
      3) The erythrocyte ruptures, the merozoites enter other red blood cells and the cycle is repeated, while the pigment is taken up by the monocytes.
      4) The length of the cycle depends on the species, see Fig. 10.

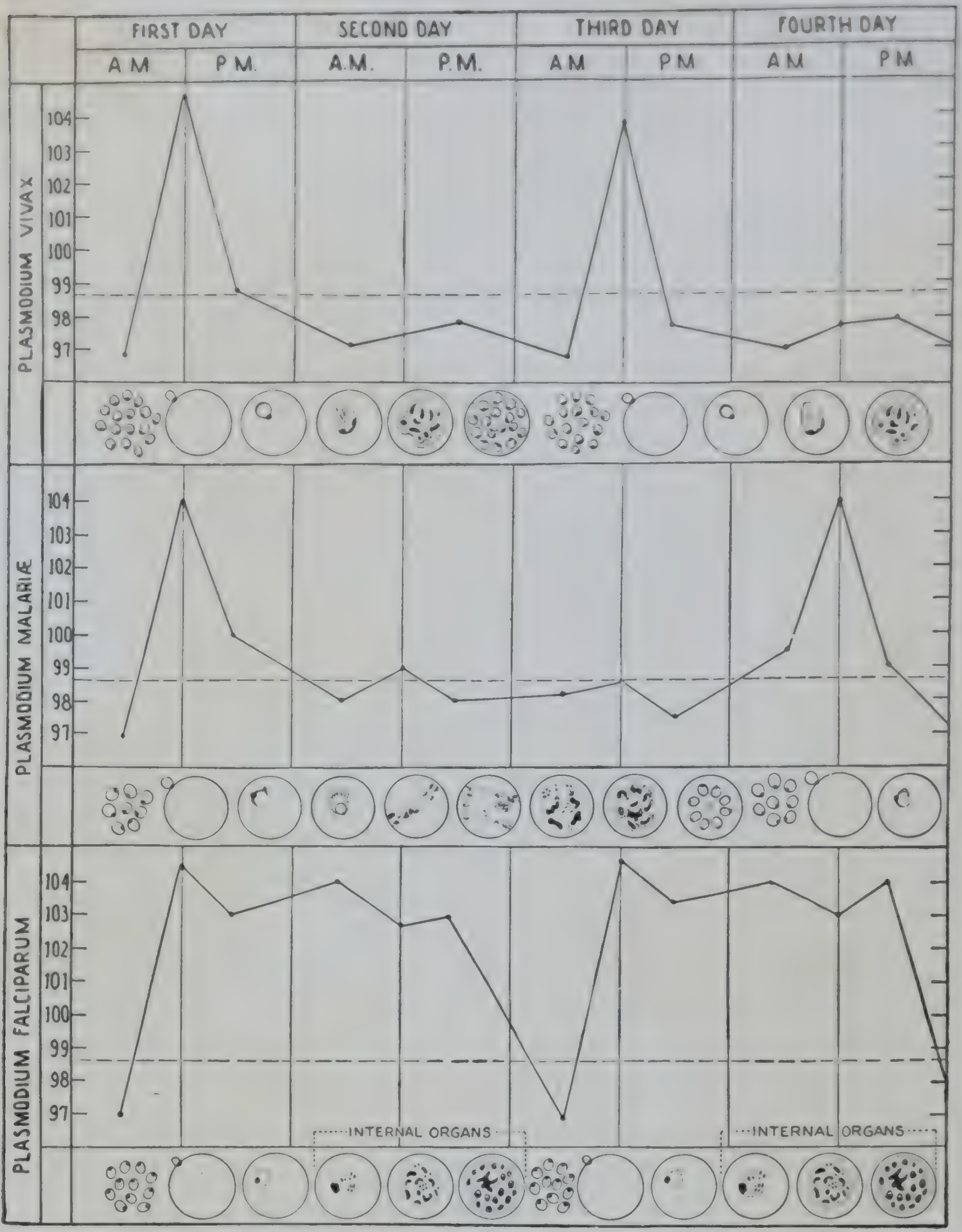


FIG. 10. TEMPERATURE CURVES IN MALARIA SHOWING RELATION TO GROWTH AND SCHIZOGONY OF MALARIAL PARASITE
(From Belding's "Textbook of Clinical Parasitology," courtesy of
D. Appleton-Century Company, New York City.)

2. *Sexual Forms in Man.*
   a. After several generations of merozoites, some develop into sexual forms called gametocytes; the male are called microgametocytes, the female, macrogametocytes.
   b. They take twice as long as asexual forms to mature in the erythrocyte and after rupture of the cell remain free in the plasma for a long time unless taken by a mosquito in feeding.
3. *Sexual Cycle in Mosquito* (Sporogony).
   a. In the stomach of the mosquito, the macrogametocyte by casting off the polar body becomes a macrogamete and the microgametocyte throws out 4-6 very motile thread-like filaments which are microgametes.
   b. The macrogamete is fertilized by a micro gamete and then is a zygote.
   c. It becomes actively motile (oökinete) an penetrates the stomach mucosa where i forms a spherical oöcyst.
   d. The oöcyst enlarges forming thousand of sporozoites.
   e. When the oöcyst ruptures, the sporozoite invade the body of the mosquito an migrate to the salivary glands from whic they are injected into man.
   f. This cycle requires 12 days.

D. *Laboratory Findings in Acute Malaria.*

1. Parasites found in thin and thick smears o blood.
2. Leukopenia with an increase in monocyte (afebrile period).

TABLE 11. PRINCIPAL DIFFERENTIAL POINTS OF MALARIA (Stained Smear)

	Plasmodium vivax (benign tertian)	Plasmodium malariae (quartan)	Plasmodium falciparum (estivo-autumnal)
Incubation period	6 to 14 days	12 to 18 days	2 to 10 days or more
Period of asexual development	48 hours	72 hours	24 to 48 hours
Infected erythrocyte	Larger than normal, paler staining, often bizarre in shape. Schüffner's dots (red granules) very often present. Multiple infection common.	Normal or slightly smaller. Sometimes brassy in appearance. Ziemann's (fine pink) dots rare. Multiple infection very rare.	Normal in size, sometimes purplish in color and occasionally a few red coarse irregular dots (Maurer's dots) are seen. Multiple infection very common.
Signet ring (young trophozoite)	Small and large rings of light blue cytoplasm. Usually one heavy red chromatin dot.	Ring smaller, thicker, heavier, and deeper blue than P. vivax. Usually one large red chromatin dot.	Rings small, delicate, and blue in color. Often two small red chromatin dots. Marginal and bridge forms are frequent.
Growing trophozoite	Cytoplasm light blue, irregular with vacuoles. Chromatin in red dots or threads. Fine yellow-brown pigment in cytoplasm.	Cytoplasm deep blue, compact, oval, band or ribbon-like. Chromatin in red dots or threads. Coarse dark-brown pigment, more abundant at periphery.	This stage remains in the ring form, the chromatin and cytoplasm increase in size. A few dark-brown granules may be present. Oldest stage seen in peripheral blood.
Schizont (presegmented form)	Cytoplasm light blue, irregular, separated into strands and particles. Chromatin divided into 2 to 10 masses. Fine yellow-brown pigment in clumps.	Cytoplasm deep blue, compact, oval or round. Chromatin divided into 6 to 8 irregular clumps. Coarse dark-brown pigment usually not clumped.	Rarely seen in peripheral blood. Resembles P. malariae but smaller. Pigment is fine dark brown and may be clumped in one small mass.
Mature schizont (segmented form)	12 to 24 divisions or merozoites composed of a dot of chromatin and a portion of cytoplasm. Pigment is in 1 or 2 clumps, usually eccentric. Parasite fills enlarged cell.	6 to 12, usually 8 or 10 merozoites in a rosette. Central mass of pigment. Parasite fills cell.	Rarely seen in peripheral blood. 8 to 24 merozoites which are very small. Central or eccentric mass of dark-brown pigment. Parasite fills about ⅔ of cell.
Macrogametocyte (female)	Spherical, deep-blue homogeneous cytoplasm with no vacuoles. Chromatin compact, dark red, and eccentric. Abundant yellow-brown pigment scattered through cytoplasm. Fills enlarged cell.	Same as P. vivax except pigment is coarser and dark brown and parasite fills a normal-sized cell.	Cresent-shaped, pointed ends, deep blue, usually a single dark-red chromatin mass near center, associated with concentrated, coarse dark-brown pigment.
Microgametocyte (male)	Spherical, light-blue, gray, pink, or almost colorless cytoplasm. Large, diffuse, light red or pink chromatin mass, usually centrally placed. Abundant yellow-brown pigment throughout cytoplasm. Smaller than macrogametocyte, about size of normal erythrocyte.	Same as P. vivax except smaller and the dark-brown pigment is usually clumped in masses.	Sausage-shaped, broader, shorter, with more rounded ends than macrogametocyte. Cytoplasm pale blue, grayish blue, or pink. Loose, diffuse, light staining particles or threads of chromatin throughout central half or more of parasite associated with numerous granules of dark-brown pigment.
First appearance of gametocytes in blood	At onset of fever.	Several months after preliminary attack.	One week after onset of fever.

Plate 6. P. vivax.

1. Normal-sized red cell with marginal ring from trophozoite.
2. Young signet ring form trophozoite in a macrocyte.
3. Slightly older ring form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling. (1)

6, 7. Very tenuous medium trophozoite forms.

8. Three amoeboid trophozoites with fused cytoplasm.

9, 11, 12, 13. Older amoeboid trophozoites in process of development.

10. Two amoeboid trophozoites in one cell.

14. Mature trophozoite.

15. Mature trophozoite with chromatin apparently in process of division.

16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").

20. Mature schizont.

21, 22. Developing gametocytes.

23. Mature microgametocyte.

24. Mature macrogametocyte.

(1) Schüffner's stippling does not appear in all cells containing the growing and older forms of P. vivax as would be indicated by these pictures, but it can be found with any stage from the fairly young ring form onward.

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PLATE 6

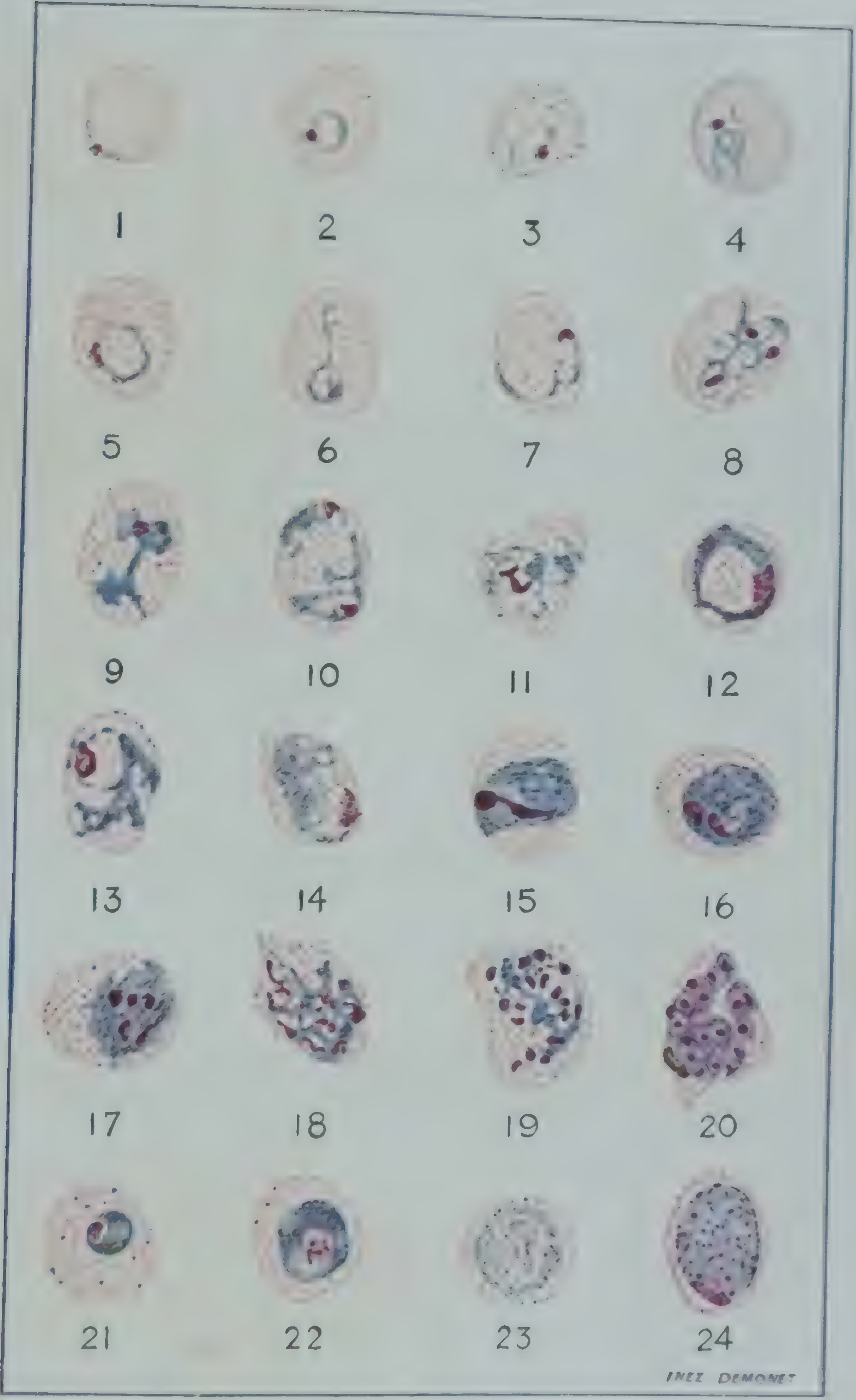
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PLATE 7. P. MALARIAE.

1. Young ring form trophozoite of quartan malaria.
2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.
5. Developing ring form trophozoite showing pigment granule.
6. Early band form trophozoite—elongated chromatin, some pigment apparent.
7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.
13, 14. Mature trophozoites—one a band form.
15, 16, 17, 18, 19. Phases in the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21. Immature microgametocyte.
22. Immature macrogametocyte.
23. Mature microgametocyte.
24. Mature macrogametocyte.

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PLATE 7

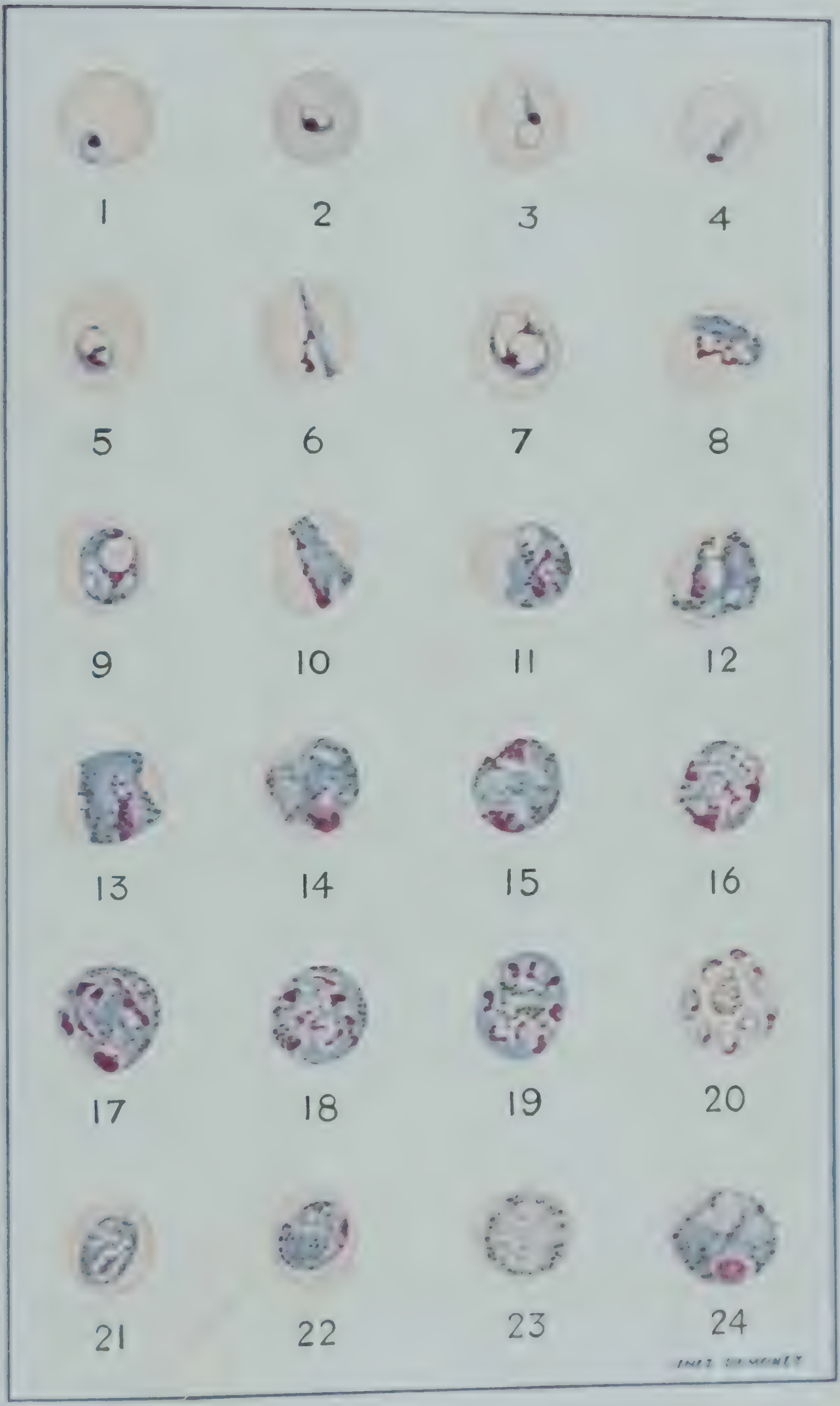
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PLATE 8. P. FALCIPARUM.

1. Very young ring form trophozoite.
2. Double infection of single cell with young trophozoites, one a "marginal form," the other "signet ring" fo
3, 4. Young trophozoites showing double chromatin dots.
5, 6, 7. Developing trophozoite forms.
8. Three medium trophozoites in one cell.
9. Trophozoite showing pigment, in a cell containing Maurer's dots.
10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.
12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's dots in the cell.
13. Estivo-autumnal "slender forms."
14. Mature trophozoite, showing clumped pigment.
15. Parasite in the process of initial chromatin division.
16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21, 22, 23, 24. Successive forms in the development of the gametocyte—usually not found in the peripheral circulati
25. Immature macrogametocyte.
26. Mature macrogametocyte.
27. Immature microgametocyte.
28. Mature microgametocyte.

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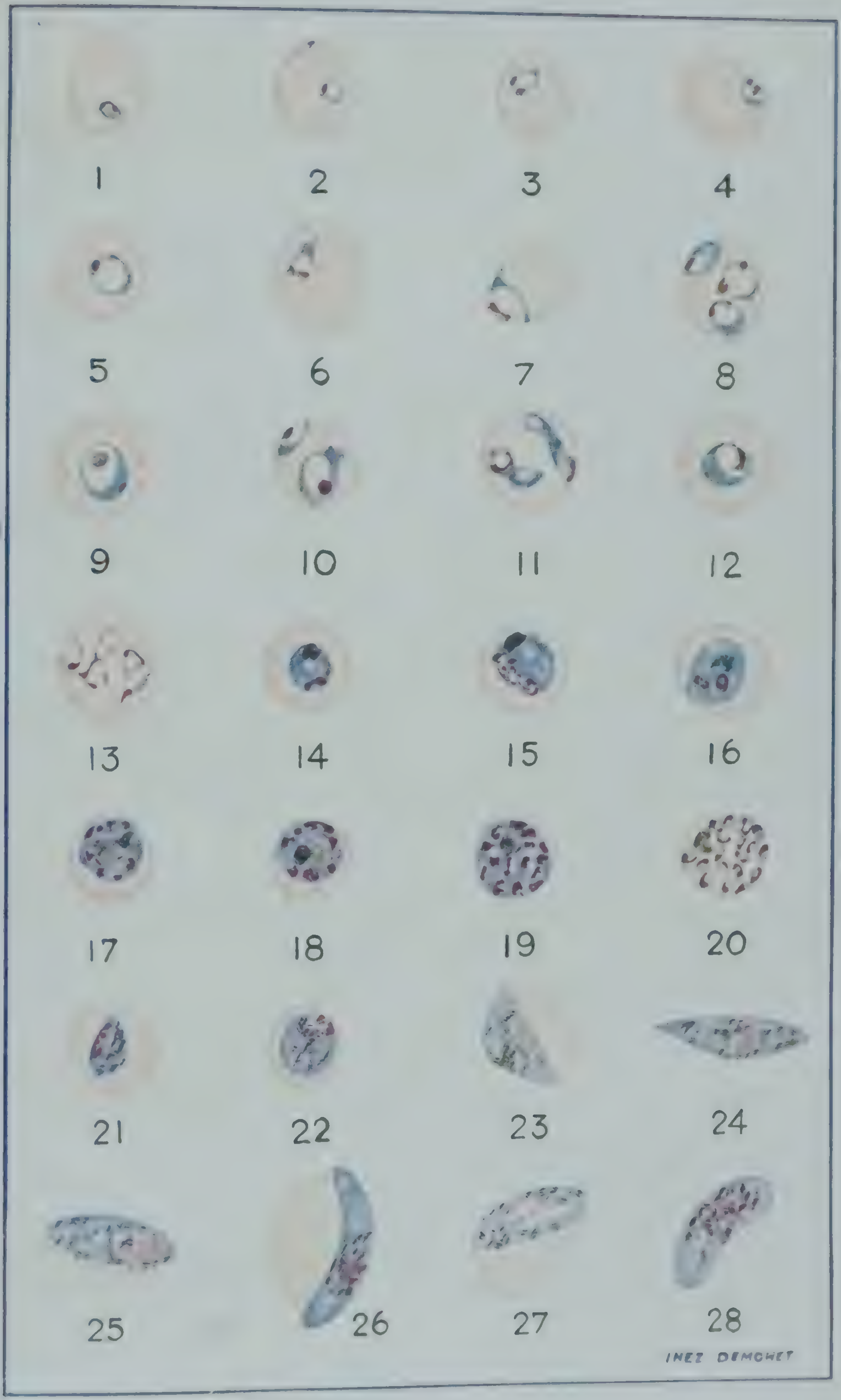
stitute of Health Bulletin No. 180, by Aimee Wilcox, U. S. Public Health Service.)

PLATE 8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3. Leukocytosis with an increase in band cells during paroxysm.
4. Malarial pigment in neutrophils and monocytes.
5. Normocytic normochromic anemia.
6. Nucleated erythrocytes present.
7. Reticulocytes increased.
8. Parasites found in bone marrow smear (sternal puncture).
9. Increased icterus index.
10. Increased urobilinogen in urine.
11. Decrease of serum protein with reversal of A/G ratio.

E. *Laboratory Findings in Chronic Malaria.*

1. Moderate leukopenia with an absolute increase in monocytes.
2. Normocytic hypochromic anemia.
3. Increase in reticulocytes.
4. Basophilic stippling in erythrocytes.
5. Parasites may be found in thick smears.
6. Parasites may be found in bone marrow smear (sternal puncture).

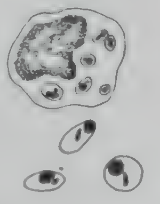
II. Leishmania.

A. *Species in Man.*

1. *L. donovani* is the cause of visceral leishmaniasis, dumdum fever, or kala-azar.
2. *L. tropica* is the cause of cutaneous leishmaniasis or oriental sore.
3. *L. braziliensis* is the cause of mucocutaneous leishmaniasis or espundia.

B. *Leishman-Donovan Body* (leishmanian form).

1. This is the only form found in the human.
2. It is a nonflagellated oval body, 2 to 4.5 microns long and 1 to 2 microns wide, found in the reticulo-endothelial cells of nearly all the internal organs, occurs only in small numbers in the monocytes and polymorphonuclear leukocytes of the peripheral blood.
3. The cytoplasm stains pale blue, the oval nucleus at the posterior end stains reddish with a delicate membrane and a large central karyosome, the rod-shaped kinetoplast is anterior to the nucleus and stains a deep violet.



C. *Leptomonas* (flagellated form).

1. The Leishman-Donovan body develops into the leptomonad or flagellated form in the invertebrate host (sand fly) or in cultures.
2. The oval body elongates and a single fla lum appears anteriorly.

D. *Diagnosis.*

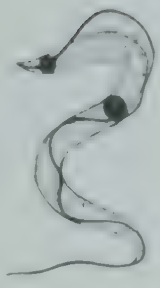
1. Stain all smears and sections with Giem stain.
2. The different species of leishmania are m phologically indistinguishable.
3. *Kala-azar.*
    a. The parasites are found in monocy and polymorphonuclear leukocytes in t or thick blood smears, but are present such small numbers that a diagnosis rarely made by this method.
    b. Smears of material obtained by punct of the sternum, spleen, or liver are amined for the parasites.
    c. Cultures of blood (sedimented cells fr oxalated blood) or aspirated splenic hepatic tissue can be made in the medi of Novy, MacNeal, and Nicolle (N.N medium).
    d. Excised enlarged lymph glands are amined by impression smears or sectio
    e. There is a leukopenia with a relative crease in lymphocytes and monocytes.
    f. Slight macrocytic hyperchromic anem
    g. Inversion of the A/G ratio of serum p teins.
    h. Cold agglutinins are sometimes found the blood.
4. *Oriental Sore and Espundia.*
    a. Smears of curettings from the base margins of ulcers are examined for pa sites.
    b. Material aspirated from the indurated m gin of a lesion under sterile conditi can be cultured in N.N.N. medium.

III. Trypanosoma.

A. *Species in Man..*

1. *T. gambiense* is the cause of African slee ing sickness.
2. *T. rhodesiense* is the cause of East Afric sleeping sickness.
3. *T. cruzi* is the cause of Chagas' disease Brazil.
4. T. gambiense and T. rhodesiense occur o in the flagellated form in man, are similar morphology, divide by binary longitudi fission, and are transmitted by the tsetse fli
5. T. cruzi occurs both in the flagellated a leishmanian forms in man and is transmitt by the reduviid bug.

Flagellated Form.



They measure 15 to 30 microns long by 1.5 to 3.5 microns wide, T. cruzi is smaller than the other two types.

They contain a nucleus, a kinetoplast, and a single flagellum bordering an undulating membrane and extending anteriorly.

In fresh preparations of blood they are actively motile.

In stained blood smears the cytoplasm is pale blue, the nucleus reddish purple, the kinetoplast dark red, and the flagellum red.

Leishmanian Form of T. cruzi.

The flagellated form does not divide by fission in the human but loses its flagellum and undulating membrane, invades endothelial or tissue cells (especially heart muscle), and then divides by binary fission producing numerous leishmanian forms within a cyst-like cavity. The leishmanian forms develop into trypanosomes and are liberated into the blood by rupture of the cyst which is accompanied by fever.

Diagnosis.

Stain all smears with Giemsa's or Wright's stain.

Make thin and thick smears of the blood during the febrile stage.

Examine unstained fresh blood for motile forms, but it is better to use a drop from the leukocyte layer of centrifuged oxalated blood.

Examine the sediment of centrifuged cerebrospinal fluid during the sleeping sickness stage.

T. gambiense and T. rhodesiense may be found in smears made of fluid aspirated from enlarged lymph glands.

Inoculate mice or guinea pigs intraperitoneally with blood, cerebrospinal fluid, or material aspirated from lymph glands during the chronic stage and examine the animal's blood daily for 2 weeks for trypanosomes.

Cold agglutinins are sometimes found in the blood.

IV. Toxoplasma.

. *Parasite.*

. Toxoplasma is a minute organism varying in size from 4 to 7 by 2 to 4 microns, having distinct cytoplasm and nuclear chromatin.

The organisms are crescentic in shape with one end pointed and the other end rounded.

3. Growing forms become oval or round prior to division by binary fission.
4. They occur singly, in clusters or aggregates within cells of the reticulo-endothelial system, parenchymal cells of the lungs, liver, adrenals, brain, kidneys, and unstriated or striated muscle.

B. *Diagnosis.*

1. Make smears of the sediment of cerebrospinal, pleural, and peritoneal fluids, sputum, and vaginal exudates.
2. Make impression smears of biopsy tissue.
3. Stain smears with Wright's or Giemsa's stain.
4. Examine the sediment of any xanthochromic ventricular or cerebrospinal fluid from infants less than 2 months old for toxoplasma. The parasite may be found in polymorphonuclear leukocytes or monocytes in cases of congenital toxoplasmosis.
5. Biopsy tissue fixed in Zenker's fluid or 10% formalin is stained with hematoxylin and eosin.
6. Suspensions of tissues in 0.85% NaCl solution or body fluids can be inoculated into mice and guinea pigs.
    a. Inject each of 6 mice with 0.03 cc. of fluid intracerebrally and 1 cc. intraperitoneally.
    b. Inject each of 2 guinea pigs with 0.2 cc. of fluid intracerebrally and about 5 cc. intraperitoneally.
    c. Observe the mice for 1 month and the guinea pigs for 6 weeks.
    d. Examine impression smears and sections of the brain and lungs of animals, that become sick or die, for Toxoplasma.
7. The complement-fixation test and the test for neutralizing antibodies are too complicated to be done in most laboratories.

V. Filaria.

A. *Diagnosis.*

1. Made by finding the adult worm or microfilaria in sections of the lesion or by finding microfilaria in the blood or chylous fluid.

2. *The adult worm* is white to creamy white, the male ranges from 2 to 70 cm. in length; the female is usually twice as long as the male.
3. *Microfilaria.*
    a. They range from 175 to 350 microns in length (see illustration).
    b. They stain with Wright's or Giemsa's stain and are found either in smears of

blood (thick or thin) or chylous fluid according to the species of filaria.

c. They are seen in fresh preparations of blood or chylous fluid by the whipping motion of the organism among the erythrocytes.

d. They may be of the periodic or nonperiodic strain.

1) Those of the periodic strain occur in greatest numbers in the peripheral blood between 8 P.M. and 2 A.M., therefore, it is best to obtain blood for examination about 10 P.M.

2) Those of the nonperiodic strain may be found in the blood at any time.

3) The microfilaria of Loa loa are only found in the day time.

e. The microfilaria, in themselves, are nonpathogenic and noninfective to man.

f. They can only complete their development by passing through the mosquito and thence back to man.

4. *Concentration Method.*

a. Obtain 5 cc. of blood from a vein and place in a centrifuge tube containing 10 cc. of distilled water.

b. Mix until the blood is laked.

c. Place 2 or 3 cotton threads in the tube and centrifuge for 5 minutes.

d. Pour off supernatant fluid, pick up threads with a rough straight piece of wire, and place on a slide.

e. Examine with the low power objective of the microscope.

f. Microfilaria, if present, will be entangled in the cotton threads.

5. Calcified worms may be seen in a roentgenogram.

B. *Wuchereria bancrofti.*

1. *Distribution:* In all tropical and subtropical countries.

2. *Location in Body.*

a. Adult worm found in the lymphatics, producing elephantiasis usually of the lower extremities and scrotum.

b. Microfilaria may be of periodic or nonperiodic strain and found in the blood and lymph.

3. *Intermediate Host:* Mosquitoes (Culex, Aedes, Mansonia, and Anopheles).

C. *Wuchereria malayi.*

1. *Distribution:* East Indies and southern Asia.

2. Microfilaria found in the blood only at night.

3. Similar in other respects to W. bancrofti.

D. *Onchocerca volvulus.*

1. *Distribution:* Africa and Central America.

2. *Location in Body.*

a. Adult worm found in the subcutane tissue forming nodules ranging from to 5 cm. in diameter.

b. Microfilaria in skin and nodules.

3. *Intermediate Host:* Black flies (Simulium a Eusimulium).

E. *Acanthocheilonema perstans.*

1. *Distribution:* Africa and S. America.

2. *Location in Body.*

a. Adult worm found in the mesentery, pe renal and retroperitoneal tissues.

b. Microfilaria are nonperiodic and found the blood.

3. *Intermediate Host:* Midges (Culicoides au tini and grahami).

F. *Loa loa.*

1. *Distribution:* Africa.

2. *Location in Body.*

a. Adult worm found in the subcutaneou tissues.

b. Microfilaria found in the blood only dur ing the day.

3. *Intermediate Host:* Tabanid flies (Chrysop dimidiata and silacea).

G. *Mansonella ozzardi.*

1. *Distribution:* South and Central America.

2. *Location in Body.*

a. Adult worm found in the mesentery an body cavities.

b. Microfilaria are nonperiodic and found i the blood.

3. *Intermediate Host:* Midges (Culicoides fu rens).

H. *Microfilaria streptocerca.*

1. *Distribution:* Africa.

2. *Location in Body.*

a. Adult worm not known.

b. Microfilaria found in the skin.

3. *Intermediate Host:* Not known.

I. *Dracunculus medinensis* (Guinea worm)

1. *Distribution:* Asia, Africa, and Arabia. Les common in West Indies and Brazil.

2. *Location in Body.*

a. Female worm is 50 to 120 cm. in lengt and migrates from the body cavities o deeper tissues when sexually mature to th subcutaneous tissues of the legs, arm shoulders, or trunk to form a sterile a scess and blister. In a short time this blis ter bursts and, if this part of the bod comes in contact with water, a loop o the uterus of the parasite prolapses throug its body wall, ruptures, and liberates larg numbers of larvae into the water.

b. Male is 12 to 29 mm. long and is rarely seen.
c. Larvae, 600 by 20 microns, are free-swimming, having a slender rhabditiform shape with a long filiform tail tapering to a sharp point.

. *Intermediate Host:* Water-flea (Cyclops).

Spirochetal Diseases

I. Relapsing Fever.

. ***Diagnosis.***

. Causative agent is a spirochete (Borrelia) 15 to 30 microns in length with 3 to 12 loose irregular coils.
. It is found in thick and thin smears of the blood stained with either Wright's or Giemsa's stain.
. It is actively motile in an unstained fresh preparation of blood.
. Examination of the blood is most successful at the height of the first pyrexial attack.
. Inoculate 0.2 to 0.5 cc. of blood intraperitoneally into white mice and examine a drop of blood from the tip of the tail daily until the 14th day.

. ***Distribution of Species.***

B. recurrentis in Europe.
B. duttoni in Africa.
B. carteri in India.
B. novyi in United States.

. ***Intermediate Host:*** Lice and ticks.

II. Leptospirosis.

. ***Etiology.***

. The causative agent is a leptospira which is a slender delicate organism 4 to 20 microns in length having a tight elementary spiral extending throughout the body. The caudal portion is bent into a hook when the organism is in motion and the anterior end is straight. The different species of leptospira are morphologically identical.
. *L. icterohemorrhagiae* causes Weil's disease or spirochetal jaundice and is transmitted by rats and dogs, usually by bites.
. *L. canicola* also causes spirochetal jaundice and is transmitted only by dogs.
. *L. grippotyphosa* causes swamp fever in Eastern Europe, the animal reservoir is unknown.
. *L. hebdomadis* causes the seven day fever of Japan and India and is transmitted by field mice or field voles.
. *L. autumnalis* causes hasami fever in Japan and is transmitted by field mice and wild rats.

B. ***Diagnosis during Febrile Stage* (1st to 5th day).**

1. Inoculate each of 2 white guinea pigs (weighing less than 300 gm.) intraperitoneally with 3 to 5 cc. of citrated blood.
   a. Examine for jaundice and take temperatures daily for 3 weeks.
   b. If a marked rise in temperature (over 103°F.) occurs after 7 to 10 days, remove 1 to 2 cc. of blood from the heart and place in an equal amount of 1% sodium citrate solution.
   c. Examine for leptospira by dark-field illumination.
   d. If organisms are found, kill animal with ether and look for jaundiced tissues and numerous petechial hemorrhages especially in the lungs and inguinal region.
   e. Stain sections of liver and kidneys with Giemsa's stain for leptospira.
2. Inoculate Noguchi's or Schüffner's leptospira medium with 0.5 cc. of blood, incubate anaerobically at 25°C. and make a dark-field examination for motile leptospira weekly for at least 4 weeks.
3. Direct dark-field examination of the blood may be made but is difficult to interpret due to the presence of artifacts.
4. The Van den Bergh test is positive in both the direct and indirect reactions.
5. The nitrogenous constituents of the blood are increased due to damage to the kidneys.
6. There is a leukocytosis with an increase in lymphocytes.
7. The urine contains albumin, casts, erythrocytes, and increased urobilinogen.

C. ***Diagnosis during Icterus Stage* (6th to 13th day).**

1. Leptospira have disappeared from the blood and agglutinins appear.
2. An agglutination test with the patients serum using either a living culture of leptospira or a formalized antigen, will be positive in final dilutions higher than 1 to 40.
   a. Mix 0.5 cc. of culture with 0.5 cc. of serum dilutions.
   b. Incubate at 37°C. for 2 hours.
   c. Make a dark-field examination for agglutination.
3. Inoculate guinea pigs, also make cultures and a dark-field examination using urine sediment instead of blood. The patient should be given enough sodium bicarbonate before collecting the urine to make it alkaline.

D. ***Diagnosis during Convalescent Stage* (13th day to recovery).**

1. Same tests as in *Icterus Stage.*

2. The agglutination test will have a higher titer.

III. Rat-bite Fever.

A. ***Sodoku.***

1. Causative agent is spirillum minus (spirochaeta morsus minus) which is 2 to 5 microns long with 3 to 4 undulations, has a rigid body and bipolar flagella.
2. Examine thick and thin blood smears stained with Giemsa's stain.
3. Inoculate guinea pigs intraperitoneally with blood, urine sediment, exudate from initial lesion, serum expressed from erythematous patches, material aspirated from lymph glands, or ground up tissue. Examine blood of guinea pigs daily for organisms.

B. ***Haverhill Fever.***

1. Causative organism is Streptobacillus moniliformis which is pleomorphic, appearing as slender filaments with spherical, oval, or fusiform swellings and tending to break up into bacillary and coccal forms. Usually stains gram-negative but young forms may be gram-positive.
2. Make blood cultures in veal infusion broth containing 20 per cent rabbit serum and incubate anaerobically.
    a. Organisms found in red blood cell sediment after 24 to 48 hours.
    b. Subculture on ascitic or Loeffler's serum medium.

Tests for Hemorrhagic Tendencies

I. **Spontaneous bleeding** may be the result of one of the following if there is no evidence of injury.

A. ***Alteration of permeability and resistance of the endothelium,*** due to:

1. Platelet deficiency which influences permeability of vascular endothelium.
2. Impaired nutrition, especially lack of ascorbic acid.
3. Toxic substances: drugs (quinine, iodides, bismuth, gold, and mercury) and products of infections (meningitis, scarlet fever, typhoid fever, smallpox, and influenza) and of metabolic origin (chronic nephritis).
4. Allergy.

B. ***Deficiency of Prothrombin.***

1. Defect in supply of vitamin K.
    a. Absence of bacteria in intestines.
    b. Inadequate supply from mother resulting in hemorrhagic disease of the newborn.
    c. Defective formation or inactivation as in sweet-clover disease of cattle.
2. Faulty absorption of vitamin K from intestinal tract due to lack of bile salts.
    a. Obstructive jaundice.
    b. Biliary fistula.
    c. Sprue (disturbed fat metabolism).
3. Faulty utilization of vitamin K due to li damage.
4. Salicylate therapy.
5. Heparin and dicumarol therapy.

C. ***Deficiency of Calcium Ions*** (rarely, if ev a cause of bleeding).

D. ***Deficiency of Thromboplastin.***

1. Diminished number of platelets—prim (idiopathic) and secondary (symptomati thrombocytopenia.
2. Decreased "plasma thromboplastin" in hem philia.

E. ***Deficiency of Fibrinogen.***

1. Nutritional deficiency.
2. Diseases of hematopoietic system.
3. Severe liver damage.
4. Snake venom.
5. Congenital defect.
6. Chloroform and phosphorus poisoning.

F. ***Presence of Heparin in the Blood.***

1. Peptone shock.
2. Anaphylactic shock.

II. Theories of Coagulation.

A. ***Howell's Theory of Blood Coagulation.***

1. Prothrombin is inactivated by antiprothro bin (heparin).
2. Thromboplastin neutralizes antiprothromb and releases prothrombin.
3. Prothrombin + calcium = thrombin.
4. Thrombin + fibrinogen = fibrin (clot).

B. ***Morawitz' Theory of Blood Coagulation.***

1. Prothrombin + calcium + thromboplastin = thrombin.
2. Thrombin + fibrinogen = fibrin (clot).

III. Coagulation Time.

A. ***General Considerations.***

1. Lee and White's venous blood method is mo accurate than the methods using capillar blood.
2. Test tubes and capillary tubes must be chem ically clean and dry.
3. Capillary tubes should be about 1 mm. i diameter and 10 cm. long.
4. Blood must not be contaminated with tissu juice.
5. A rough estimation of coagulation time ca be made when drawing blood for serolog tests by using a modified Howell's metho

Lee and White's Venous Blood Method.

1. Use a 5 cc. sterile syringe and needle that has been washed out with sterile 0.85% NaCl solution. The last of the NaCl solution is expelled with the needle held vertically, so that the needle and dead space in the tip of the syringe are left filled.
2. Withdraw quickly 4 cc. of blood from a vein, recording the time of the first appearance of blood in the syringe. Avoid getting air bubbles in the syringe.
3. Remove needle from syringe and place 1 cc. of blood in each of 3 chemically clean test tubes (8 mm. in diameter) which have been rinsed with 0.85% NaCl solution just before adding the blood.
4. After 4 minutes tilt the first tube slightly to determine whether coagulation has taken place.
5. Thirty seconds later examine the second tube in the same way.
6. Alternately tilt the 1st and 2nd tube at 30 second intervals until coagulation has taken place.
7. Then test the third tube in the same manner; record the time at which this tube can be inverted without disturbing the clot.
8. The time elapsed since the first appearance of blood in the syringe and clot formation in the third tube is the coagulation time.
9. The normal is 5 to 10 minutes.
10. If the diameter of the tube is 9 mm., the normal is 6 to 13 minutes.

Capillary Blood Methods.

Method of Obtaining Blood.

a. Blood may be taken either from the ear or finger.
b. The puncture should be deep enough to insure free flow of blood in order to decrease contamination with tissue juice.
c. The first 2 drops should be wiped off and the third used for the test.
d. The coagulation time is counted from the first appearance of the drop of blood used.

Capillary Tube and Hair Method.

a. Insert a white horse hair (previously washed in ether) in a capillary tube.
b. Place one end of the tube in a drop of blood and let it fill one-half full by capillary action.
c. After 1 minute draw the horse hair out half an inch from the end filled with blood; repeat at 30 second intervals until clumps of fibrin adhere to the hair.
d. The coagulation time is the interval between the first appearance of the drop of blood to the first adherence of fibrin to the hair.
e. Normal 2 to 6 minutes.

3. *Capillary Tube Method.*
   a. Place one end of each of 2 capillary tubes in a drop of blood and let the tubes fill simultaneously by capillary action for about two thirds of their length.
   b. Lay the tubes on the table.
   c. After 1 minute pick up 1 tube and very gently break off 1 centimeter of the filled end.
   d. Repeat at 30 second intervals until coagulation has occurred; this is revealed by the presence of strands of fibrin which span the gap at least 5 mm. between the broken ends.
   e. Care must be taken not to break the fibrin when breaking the tube.
   f. Immediately confirm by breaking the second tube.
   g. Usually the handling of the first tube will decrease the time of coagulation.
   h. The end-point is the time elapsed from the appearance of the drop of blood and coagulation in the second tube.
   i. Normal 2 to 6 minutes.
4. *Slide Method.*
   a. Place 2 separate large drops (4 to 5 mm.) of blood on a clean slide.
   b. Draw a pin through one at 30 second intervals.
   c. As soon as the fibrin thread can be pulled up by the pin, coagulation has taken place.
   d. Check with the other drop to see if coagulation has taken place.
   e. Take the time of coagulation of the second drop for the final result if it is longer than the first.
   f. Normal 2 to 6 minutes.

D. *Howell's Method* (Modified).

1. Rinse a sterile 10 cc. syringe with sterile 0.85 NaCl solution in the same manner as described under the Lee and White's method.
2. Draw venous blood and take the time of the first appearance of the blood in the syringe.
3. Place 7 cc. in a test tube 5/8 inch in diameter.
4. At 10 minute intervals tip the tube to see if coagulation has taken place.
5. The end-point is when the tube can be inverted without disturbing the clot.
6. Normal 10 to 40 minutes.

E. *Abnormal Coagulation Time.*

1. Prolonged in hemophilia (see Table 12), occasionally in obstructive jaundice, anemias, leukemias, hemorrhage of the newborn, and at the onset of severe, acute fevers.
2. Decreased in digitalis medication.

IV. Bleeding Time.

A. *General Considerations.*

1. Bleeding time does not necessarily parallel the coagulation time of the blood. It is chiefly dependent upon the efficiency of the tissue juice (thromboplastin) in accelerating clotting, upon capillary contractility, and upon the mechanical and chemical action of the blood platelets.
2. If the bleeding time is prolonged, do not continue test longer than 20-30 minutes.

B. *Venostasis Bleeding Time* (Ivy's Method).

1. Wash the lateral part of the forearm near the elbow gently with 70% alcohol.
2. After any hyperemia occurring from the alcohol has disappeared, place the cuff of a blood pressure apparatus on the arm above the elbow and raise the pressure to 40 mm. of mercury to prevent venous return.
3. Make a puncture in the clean area of skin with a mechanical stylet set at a depth of 3 mm. but do not puncture near a vein.
4. Remove the drops of blood with filter paper every 10 seconds until no more blood appears, being careful not to touch the skin.
5. If no bleeding occurs, this is not recorded and the blood pressure cuff is decompressed.
6. Repeat the procedure after 5 minutes and keep repeating at 5 minute intervals until there are 3 recordings of bleeding.
7. Take the average of the three bleeding times.
8. Normal bleeding time is 2 to 125 seconds.

C. *Duke's Method.*

1. Make a deep puncture in the ear as for a blood count.
2. The size of the wound should be such that the blot of blood after the first 30 seconds is 1 to 2 cm. in diameter.
3. Note the time the first drop of blood appears.
4. Remove the drops of blood with filter paper at 30 second intervals, being careful not to touch the skin.
5. The end-point is reached when no more blood appears.
6. Normal bleeding time is 1 to 3 minutes.
7. If moderately prolonged, the twentieth drop will be about one half the size of the first.
8. If markedly prolonged, the twentieth drop will be as large as the first.

D. *Prolonged Bleeding Time* (See Table 12).

1. *When blood platelets are greatly reduced.*
    a. Thrombocytopenic purpura.
    b. Acute leukemia.
    c. Aplastic anemia.
2. *Injury of capillary wall.*
    a. Scurvy.
    b. Toxins (infectious, chemical, snake v om).
    c. Allergy.
3. *Prothrombin deficiency.*
    a. Destructive diseases of the liver w hemorrhagic tendencies.
    b. Hemorrhagic diseases of the newborn.
4. *Slightly prolonged in severe anemias.*

V. Capillary Resistance Test.

A. *Tourniquet Method* (Rumpel-Leede Phenomenon).

1. Mark a definite area (5 cm. in diamet on the forearm, the upper edge of which 4 cm. below the bend at the elbow.
2. Count the petechiae already present in t area.
3. Place a blood pressure cuff on the arm a raise the pressure to 100 mm. of mercury midway between systolic and diastolic pr sure) and leave for 8 minutes.
4. Remove the cuff and after 5 minutes cou the number of petechiae in the marked ar
5. Normally there should be no more than or at the most 20 spots.
6. See Table 12.

B. *Interpretation.*

1. A positive test indicates capillary permeab ity and fragility.
2. It is positive in purpura and scurvy.

VI. Blood Clot Retraction Time.

A. *General Considerations.*

1. There is no connection or parallelism betwe the coagulation time and the retraction the clot.
2. The degree of retraction parallels the numb of platelets.
3. See Table 12.

B. *Method.*

1. Secure 5 cc. of blood by venous puncture a place in a sterile, chemically clean, grad ated centrifuge tube (note exact amount blood).
2. Stopper with cotton and place in the inc bator (37°C.).
3. Observe at 1, 18, and 24 hours.
4. If there is no retraction of the clot in 18 hou run an inoculating wire once around the i side of the tube as close to the glass as pos ble. Occasionally normal blood does not r tract but will within a short time after bei separated from the wall of the tube.
5. Remove clot by means of a mouse-tooth fo ceps and measure the volume of serum.

a. Report volume of serum in percentage of original blood volume.
b. Note the shape, firmness, and fragility of the clot.

C. *Degree of Retractility.*

Normally the coagulum commences to retract within the first hour and forms a firm clot with complete retraction in 24 hours.

The normal clot is firm, difficult to break up with blunt instruments, and difficult to flatten out. Defective clots are soft, friable, and easily crushed.

In thrombocytopenic purpura a coagulum is formed in the normal time, but it does not retract.

In hemophilia the coagulum forms very slowly, but the clot when once formed retracts normally.

D. *Presence or Absence of Digestion of Clot.*

Normally there should be no digestion of clot.

Digestion of clot, giving it a worm-eaten appearance, is seen in cirrhosis of the liver.

E. *Color and Character of the Serum.*

Normally a slight margin of clear pale yellow serum appears within the first hour and increases in amount. In 18 to 24 hours the volume varies from 40 to 60 per cent of the total amount of blood. When the clot is defective and incapable of proper retraction, the volume of serum is less than 40 per cent.

May appear milky after meals or in diabetes and leukemia.

May be deep yellow in hemolytic jaundice, in obstructive jaundice, or in carotenemia.

VII. Counting Blood Platelets (Thrombocytes).

A. *General Considerations.*

The chief function of the platelets is to control coagulation and bring about retraction of the clot.

They are the chief source of prothrombin and the probable source of a part of the thromboplastic substance of the tissues.

They are important in protecting the walls of blood vessels when the endothelium becomes injured, because they adhere to the injured surface.

B. *Indirect Method.*

1. *Platelet Solution.*

Sodium citrate	1.5 gm.
Distilled water	50.0 cc.
Formalin, neutral to litmus	2.5 cc.

a. Keeps one month in the refrigerator.
b. Filter before using.

2. Place a small amount (about 10 drops) of platelet solution in a depression 2 cm. in diameter and 0.5 cm. deep in a paraffin block.
3. If the patient's hand is cold, place in warm water for a few minutes.
4. Wash the finger tip with soap and warm water and then alcohol and acetone.
5. Cover with a thin layer of sterile vaseline.
6. Make a deep prick in the finger through the vaseline and shake the first drop of blood off the finger.
7. Immediately place the finger tip in the solution in the paraffin block and let the second and third drops of blood mix with the solution without coming in contact with the air.
8. The blood will settle to the bottom of the solution.
9. Draw up platelet solution in a leukocyte pipette to the 11 mark and then blow it out; this wets the inside of the pipette with the solution.
10. Draw up the blood from the bottom of the depression until the bulb of the pipette is about one-half full, then raise the pipette into the clear solution and fill it to the 11 mark (need not be exact).
11. The proportion of blood to solution should be about 1:3.
12. Mix the blood and the solution by shaking the pipette for 1 minute.
13. Let 3 drops run out, then make 4 thin smears. (The smears should be made immediately because, if the blood is allowed to stand in the pipette, the platelets have a tendency to clump.)
14. Stain with Wright's stain, leaving stain on 1 minute and adding buffer solution for 2 minutes.
15. Also make an erythrocyte count on the patient.
16. With a Whipple eyepiece micrometer in the microscope, or a piece of paper as described under reticulocyte count, count all the erythrocytes and platelets in 10 different areas on each of the four smears or until at least 1000 erythrocytes are counted.
17. The platelets appear as reddish to violet bodies, 2 to 4 microns in diameter, and take numerous shapes; round, ovoid, elliptical, or tennis racket shape with a long handle.
18. Find the per cent of platelets by dividing the number of platelets by the number of erythrocytes counted. Then multiply the patient's erythrocyte count by this per cent to find the number of platelets per c. mm.

C. *Interpretation* (See Table 12).

1. *Normal Count*—200,000 to 400,000 per c. mm.
    a. Varies considerably from day to day.
    b. Increases at high altitudes.
    c. Higher in winter than in summer.
2. *Decreased* in pernicious anemia, aplastic anemia (below 100,000), chronic lymphocytic leukemia, all the acute leukemias, thrombocytopenic purpura (below 60,000), cirrhosis of the liver, and at the onset of severe acute fevers, as in diphtheria, pneumonia, malaria, and typhoid.
3. *Increased* in chronic myelocytic leukemia, erythremia (polycythemia vera), chlorosis, sickle cell anemia, chronic diseases associated with cachexia and malnutrition, in some acute infections as erysipelas, septicemia, acute articular rheumatism, and after hemorrhage, splenectomy, tissue injury, and bone fractures.

TABLE 12. BLOOD FINDINGS IN SPONTANEOUS BLEEDING

Disease	Blood Platelets	Coagulation Time	Bleeding Time	Clot Retraction	Prothrombin Time	Tourniquet Test
Purpura						
Thrombocytopenic—Primary . . .	Decreased	Normal	Prolonged	No retraction	Normal	Positive
Secondary (1). .	Decreased	Normal	Prolonged	No retraction	Normal	Positive
Nonthrombocytopenic (2). . .	Normal	Normal	Normal	Normal	Normal	Positive
Thrombasthenia (3)	Normal	Normal	Prolonged	Normal or delayed	Normal	Positive
Hemophilia.	Normal	Prolonged	Normal (small incisions)	Normal	Normal	Negative
Jaundice.	Normal	Normal	Normal	Normal	Prolonged	Negative
Hemorrhage of the newborn.	Normal	Prolonged	Prolonged	Normal	Prolonged	Negative
Abnormal Gross Bleeding.	Normal	Normal	Normal	Normal	Normal	Negative

(1) Acute leukemia, Banti's syndrome, aplastic anemia, drugs, infections, etc.
(2) Allergy, drugs, infections, kidney disease, scurvy, etc.
(3) Also called hereditary hemorrhagic diathesis, hereditary hemorrhagic thrombasthenia, hereditary pseudohem philia, and constitutional thrombopathy.

VIII. Prothrombin Time (Quick's Method).

A. *Principle:* It is assumed that clotting time depends directly on the amount of thrombin present and that prothrombin activity can be measured by its ability to form thrombin. An excess of tissue extract (thromboplastin) and calcium is added to plasma to initiate thrombin formation, and the time for clot formation is considered a direct index of prothrombin concentration.

B. *Method.*

1. Keep a 5 cc. syringe and 0.1 M sodium oxalate solution in the refrigerator, so they will be cold when used.
2. Place a sterile needle on the syringe and quickly draw 4.5 cc. of blood into syringe by venipuncture. Care must taken that the vein is cleanly entered and tissue gets in the needle.
3. Place the blood immediately in a test tu containing 0.5 cc. of the sodium oxalate so tion and mix by placing the thumb over end of the tube and inverting 3 or 4 tim
4. Centrifuge blood at once at a low rate speed for 5 minutes.
5. Pipette the plasma into another test tu
6. Place a test tube containing some 0.025 calcium chloride solution (depending number of tests to be run) in a 37.5°C. wa bath to warm.
7. To 0.1 cc. of plasma in a test tube 3/8 in in diameter, add 0.1 cc. of thromboplast and mix.
8. Place in the water bath for 1 minute.
9. Blow 0.1 cc. of the warmed calcium chlori solution into the test tube and the instant is added start a stop watch.
10. Keep in the water bath and shake light to within a few seconds of the expect clotting time.
11. Hold the tube in front of a source of lig and keep tilting the tube very gently; at th first appearance of a fibrin web, stop th watch immediately. Do not shake the tu or the first fibrin mesh will be broken an an error made in the prothrombin time.
12. Record time of the fibrin web formatio as the prothrombin time.
13. Repeat once or twice to check results.
14. Repeat using plasma obtained in a simil manner from a normal person as a contr

5. Report the prothrombin time in seconds and also as per cent concentration in plasma according to the following formula:

$$\frac{N - 8.7}{P - 8.7} \times 100 = \text{per cent concentration in plasma.}$$

N = prothrombin time of normal person in seconds.
P = prothrombin time of patient in seconds.

Solutions.

Sodium Oxalate Solution— 0.1 M.
Dissolve 1.34 gm. of anhydrous sodium oxalate, c.p., in 100 cc. of distilled water.

Calcium Chloride Solution— 0.025 M.
Dissolve 1.11 gm. of anhydrous calcium chloride, c.p., in 400 cc. of distilled water.

Thromboplastin.

a. Obtain a brain from a rabbit after killing it by injecting 20 cc. of air into its ear vein.
b. Remove all blood vessels from the brain.
c. Place the brain in a mortar and cover with acetone to a depth of about an inch.
d. Grind the brain until it becomes a granular powder, replacing the acetone several times. This should not take longer than 10 minutes.
e. Dry by suction using a Buchner funnel and then regrind without using acetone.
f. Weigh the dried brain and add 0.85% NaCl solution in the proportion of 5 cc. to each 0.2 gm. of brain powder.
g. Mix the solution by blowing through a pipette and incubate for 15 minutes in a water bath at 50°C. Mix during the incubation by blowing into the tube at 7 and 14 minutes.
h. Centrifuge at low speed for 10 minutes and pour the milky supernatant fluid which is the thromboplastin into another tube.
i. The thromboplastin solution will remain stable for several weeks if kept in the coldest part of the refrigerator.
j. The thromboplastin must give a clotting time between 11 and 15 seconds with normal plasma or it must be discarded.
k. The dried brain obtained in step e may be kept in a desiccator in the refrigerator. Regrind it without using acetone before making the thromboplastin solution.

Interpretation of Prothrombin Time (See Table 12).

Normal prothrombin time: 11 to 15 seconds.

Prothrombin concentration of 70 per cent or more is considered normal.

Values below 60 per cent indicate a tendency to bleed.

Spontaneous bleeding occurs when the prothrombin concentration is less than 20 per cent.

5. Values between 20 and 30 per cent are desirable during heparin and dicumarol therapy.
6. *Increased values* (decreased per cent) are due to:
   a. Inability of the liver to form prothrombin from vitamin K in severe liver damage.
      1) The response of the prothrombin time to parenteral vitamin K therapy is not only of diagnostic but also of prognostic value in liver disease.
      2) If the prothrombin time returns to normal after vitamin K therapy, the liver is not severely damaged.
   b. Poor or no absorption of vitamin K from the intestine.
      1) Chronic lesions of the intestinal tract.
      2) Absence of bile in obstructive jaundice and external biliary fistula of long standing.
   c. Inadequate intake of vitamin K in deficiency diseases.
   d. Inability of the newborn to manufacture vitamin K because of lack of intestinal bacteria.
   e. Salicylate therapy.
   f. Heparin and dicumarol therapy.
7. *Decreased values* (increased per cent) are found in acute thrombophlebitis, multiple myeloma, after digitalis therapy, and after ether anesthesia.

Sedimentation and Fragility of Erythrocytes

I. Sedimentation Rate.

A. *Principle:* Sedimentation is due to changes in the surface charge of the erythrocytes which cause them to aggregate. These changes are related to alterations in the plasma, particularly in the physical state of the plasma colloids (Wintrobe).

B. *General Considerations.*

1. An anticoagulant must be used that will not shrink the erythrocytes.
2. The correct proportion of anticoagulant and blood must be used.
3. There must not be any clots present in the blood.
4. Do not let the blood stand more than 1 hour before starting test.
5. Blood should be at room temperature at beginning of test.
6. To avoid frothing, shake the blood gently for 3 minutes before starting the test.
7. Sedimentation tube must be chemically clean and dry.
8. There should be no bubbles in the column

of blood in the tube.

9. Tube must be in an absolutely perpendicular position and not in a draft during test.

C. ***Westergren's Method.***

1. See general considerations.
2. Draw 5 cc. of venous blood with a dry needle and syringe. The tourniquet should be applied only at the last minute so that venous stasis is of short duration.
3. Place the blood in a small bottle in which there is the correct amount of dried anticoagulant (ammonium and potassium oxalate).
4. Mix the contents at once by shaking the bottle.
5. Just before doing the sedimentation test, mix the blood thoroughly by shaking the bottle gently for 3 minutes.
6. Draw blood to the zero point of a chemically clean Westergren pipette divided into 200 mm. and place tube in a Westergren rack.
7. The room temperature during the sedimentation should be about 20°C.
8. Read the calibrations on the pipette at the upper level of the sedimentating corpuscles at the end of 1 hour.
9. Normal sedimentation rate for 1 hour.
    a. Men: 0-9 mm.
    b. Women and girls: 0-15 mm.
    c. Children under 3½ years of age: 0-20 mm.
    d. Persons over 65 years of age: 0-20 mm.
10. The values obtained by this method should not be corrected for anemia.

D. ***Wintrobe and Landsberg's Method.***

1. See general considerations and collect blood as described under Westergren's method (2 to 5).
2. Fill a chemically clean Wintrobe tube with well-mixed oxalated blood to the 10 mark as directed under cell pack (page 41).
3. Place the tube in a rack in an absolutely perpendicular position and keep at room temperature out of drafts; note the temperature of the room.
4. Read the length of the plasma layer in mm. and if the temperature of the room is not 20°C., correct the sedimentation rate according to Fig. 11.
5. If the reading is more than normal, place rubber cap over tube and centrifuge at 3000 revolutions per minute for 30 minutes. Note level of packed corpuscles and, if not normal, correct the sedimentation rate according to Fig. 12 on page 90.
6. For cleaning tube see cell pack (page 41).
7. Normal sedimentation rate for women a girls is 0 to 20 mm.; for men 0 to 9 mm.

E. ***Interpretation of Sedimentation Rate.***

1. Increased in patients with an increased brea down of tissue, such as, acute infectious di eases, chronic granulomatous infections, ca cinoma, acute heavy metal poisoning, n phritis, nephrosis, and gout.
2. Increased in rheumatic fever, rheumatic hea disease, rheumatoid arthritis, and corona thrombosis.
3. Increased in pregnancy after the 12th wee Abortion at anytime results in an increase.
4. The rate is slowed in hemolytic jaundice ar sickle cell anemia due to the change in sha of the erythrocytes.
5. The rate is normal in infectious mononucl osis.

II. Fragility of Erythrocytes.

A. ***Principle:*** The erythrocytes are placed i varying dilutions of 1 per cent sodium chl ride solution, the resultant swelling an hemolysis in the lower dilutions are an inde of the resistive power of the cells to hy potonic sodium chloride solution.

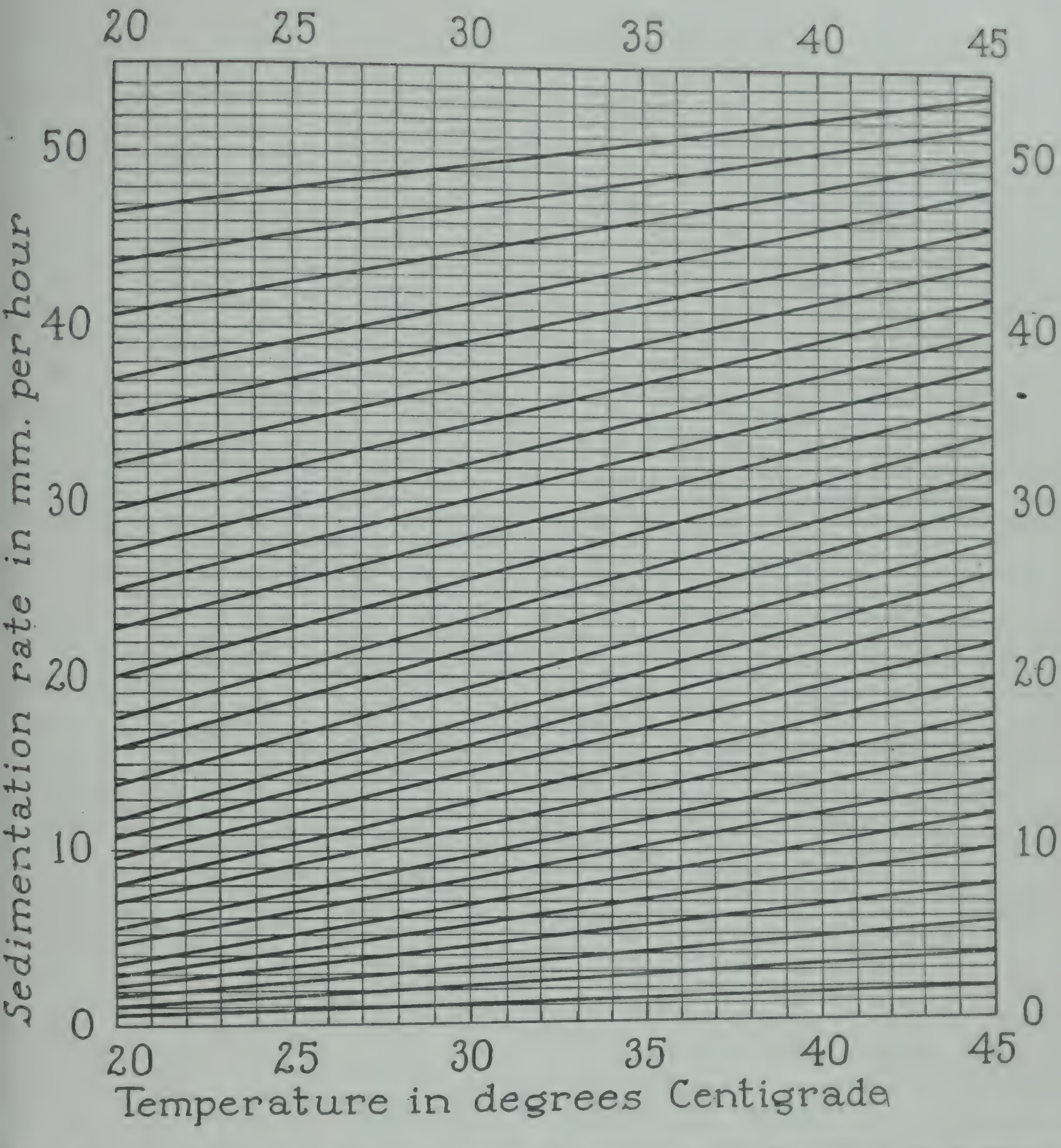
TABLE 13. FRAGILITY TEST

Tube	1% NaCl solution in cc.	Distilled water in cc.	NaCl dilution in per cent	Hemolysis
1	0.56	1.44	0.28	++++
2	0.60	1.40	0.30	++++
3	0.64	1.36	0.32	++++
4	0.68	1.32	0.34	++++
5	0.72	1.28	0.36	+++
6	0.76	1.24	0.38	++
7	0.80	1.20	0.40	++
8	0.84	1.16	0.42	+
9	0.88	1.12	0.44	0
10	0.92	1.08	0.46	0
11	0.96	1.04	0.48	0
12	1.00	1.00	0.50	0
13	1.04	0.96	0.52	0
14	1.08	0.92	0.54	0
15	1.12	0.88	0.56	0
16	1.16	0.84	0.58	0
17	1.20	0.80	0.60	0
18	1.24	0.76	0.62	0
19	1.28	0.72	0.64	0
20	1.32	0.68	0.66	0

* ++++ = complete hemolysis
0 = no hemolysis

B. ***Method.***

1. Prepare a 1% solution of sodium chlorid in a volumetric flask. Use chemically pur and freshly dried sodium chloride and weig with an analytical balance.
2. Arrange 2 series of 20 small test tubes in rack.



IG. 11. CHART FOR CORRECTION OF SEDIMENTATION RATE FOR VARIATION IN ROOM TEMPERATURE (W. B. Wartman, Am. J. Med. Sci., courtesy of Lea and Febiger, Philadelphia).

o correct sedimentation rate:

. Follow the vertical line representing the temperature of the room to the horizontal line representing the sedimentation rate.
. Follow the nearest sloping line to the left hand side of the chart to obtain the corrected value at 20°C.

3. With 1 cc. pipettes graduated in hundredths, pipette the sodium chloride solution and distilled water into the first series of 20 small test tubes according to Table 13. In certain diseases more tubes containing either lesser or greater dilutions will be necessary, see Table 14.
4. The 2 solutions in the first tube are thoroughly mixed by drawing up in a 1 cc. pipette and blowing back into the tube, repeating several times. Transfer 1 cc. of this solution to the first tube in the second series.
5. Repeat this procedure for each tube in the series, but before starting the next tube be

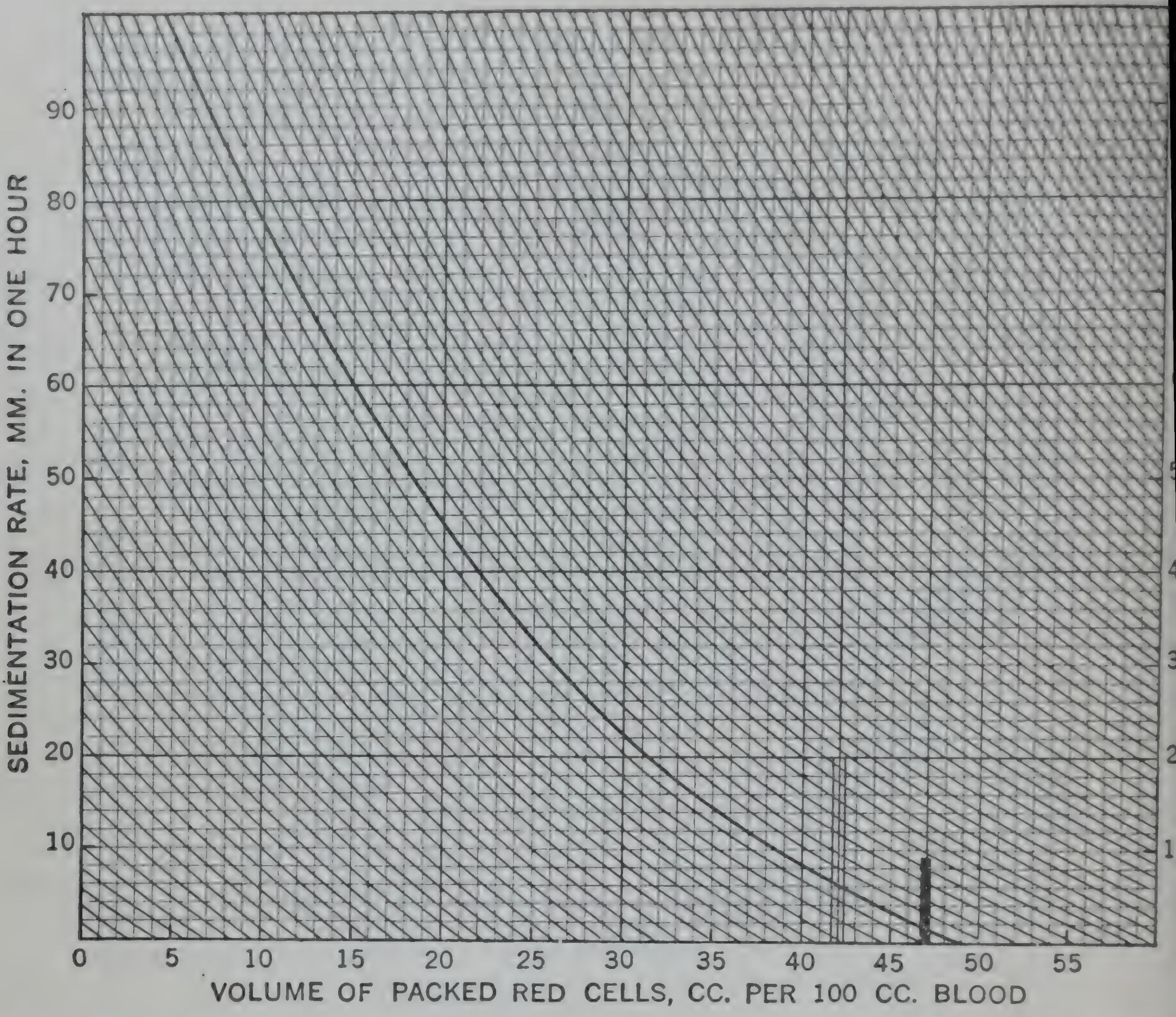


Fig. 12. Chart by Wintrobe and Landsberg for Correction of Sedimentation Rate According to Volume Packed Red Blood Cells (From Wintrobe's "Clinical Hematology," courtesy of Lea and Febiger, Philadelphi

The mean normal volume of packed erythrocytes for men (47 cc.) and for women (42 cc.) are heavily outlined. The range of normal sedimentation is represented by a solid column for men and an open column for women.

To correct sedimentation rate:

a. Follow the horizontal line which represents the sedimentation rate across the chart until it intersects the verti line which represents the volume of packed red cells.
b. Follow the nearest curved line until it intersects the heavy line at 42 cc. if the patient is a woman, or at 47 if the patient is a man.
c. At this point of intersection, read the value of the horizontal line which is the corrected sedimentation rate.

sure that all the solution is blown out of the pipette.

6. Obtain about 2 cc. of the patient's blood from a vein with a dry syringe and No. 21 needle and immediately expel 1 drop into each of the first series of 20 tubes. Each drop must be the same size.
7. Mix by shaking each tube gently. *Do not* invert with thumb over the mouth of the tube.
8. To the second series of 20 tubes add, in a similar manner, blood from a normal person.
9. Let the tubes stand 2 hours at room temperature, at which time the corpuscles will ha settled to the bottom and hemolysis may recognized by the color of the supernata fluid.
10. Initial hemolysis is recognized by a fai yellow color in the supernatant fluid.
11. Hemolysis is complete when the supe natant fluid is red and no erythrocytes c be seen in the bottom of the tube wh viewed from above.
12. Report the per cent of sodium chloride sol tion showing initial and complete hemoly in both the patient's blood and normal bloo

Interpretation.

See Table 14 for values of normal and pathological blood.

This test is essentially an index of cell shape; the spherocytes show decreased resistance, the poikilocytes increased resistance to hemolysis.

It is an important test in hemolytic jaundice. The fragility of the cells varies in the anemias according to the severity of the anemia.

TABLE 14. VALUES FOR FRAGILITY TEST

	Initial hemolysis in per cent of NaCl	Complete hemolysis in per cent of NaCl
ormal	0.46 - 0.40	0.36 - 0.30
emolytic Jaundice	0.86 - 0.52	0.48 - 0.38
lastic Anemia	0.52 - 0.46	0.32 - 0.26
rnicious Anemia	0.50 - 0.44	0.32 - 0.26
ypochromic Anemia	0.52 - 0.48	0.28 - 0.18
ooley's Anemia	0.54 - 0.38	0.20 - 0.00
ckle Cell Anemia	0.46 - 0.40	0.22 - 0.00
ronic Lymphocytic Leukemia	0.50 - 0.44	0.36 - 0.30
ronic Myelocytic Leukemia	0.60 - 0.50	0.34 - 0.26

Blood Findings in Disease

I. Interpretation of a Leukocyte Count.

Unfavorable Signs.

Extreme leukocytosis with high percentage of neutrophils.
Failure to develop leukocytosis.
High proportion of immature cells.
Numerous toxic forms.
Marked absolute reduction of lymphocytes.

Blood Picture During Recovery.

Decrease in total leukocyte count and neutrophils.
Decrease of immature forms.
Temporary increase of monocytes.
Increase of eosinophils.
Increase of lymphocytes.
Absence or decrease of toxic forms.

II. Leukopenia.

Leukopenia is a decrease in the total number of leukocytes. It is often associated with a decrease in polymorphonuclears, resulting in a relative lymphocytosis.

Diseases Producing Leukopenia.

Infections.
   a. *Bacterial*—typhoid, paratyphoid, and brucellosis.
   b. *Viral*—influenza, measles, rubella, and psittacosis.
   c. *Protozoal*—malaria, relapsing fever, and kala-azar.
2. *Overwhelming infections*—generalized miliary tuberculosis and septicemia.
3. *Hemopoietic disorders*—pernicious anemia, aplastic anemia, chronic hypochromic anemia, Banti's syndrome, Gaucher's disease, myelophthisic anemia, aleukemic leukemia, and agranulocytosis.
4. *Chronic intoxications*—lead, mercury, arsenic, gold, benzol, amidopyrine, dinitrophenol, sulfonamides, barbiturates, morphine, ether, and alcohol.
5. Cachectic and debilitated states and inanition.
6. Overdose of Roentgen ray or radium.
7. Anaphylactoid shock and early reaction to foreign protein.
8. *Miscellaneous*—myxedema, lymphosarcoma, infectious hepatitis, cirrhosis of the liver, Felty's syndrome, and lupus erythematosus disseminata.

III. Leukocytosis.

A. ***Leukocytosis*** is a temporary increase in the total leukocytes or in the percentage of a certain type, usually due to a specific stimulus, such as an infection.

B. ***Neutrophilia.***

1. *Acute infections* due to cocci, especially in pneumonia, scarlet fever, and meningitis.
2. *Localized infections* with accumulations of pus, especially when it is under pressure.
3. *Certain general infections,* such as rheumatic fever, diphtheria, and smallpox.
4. *Intoxications.*
   a. Metabolic—uremia, diabetic acidosis, eclampsia, and gout.
   b. Poisoning by certain chemicals and drugs.
5. *Hemorrhage*—acute or sudden hemolysis of erythrocytes.
6. *Postoperative.*
7. *Coronary thrombosis.*
8. *Malignant neoplasms* when growing rapidly, especially in the gastro-intestinal tract, liver, or bone marrow.
9. *Physiological* during late pregnancy and labor, after strenuous exercise, after repeated vomiting, convulsions, paroxysmal tachycardia, accompanying severe pain, in the newborn, and in a moribund condition.

C. ***Eosinophilia.***

1. *Allergic conditions*—bronchial asthma, hay fever, urticaria, serum sickness, and angioneurotic edema.
2. *Skin diseases*—pemphigus and dermatitis herpetiformis.

3. *Parasitic diseases* — trichinosis, echinococcal disease, schistosomiasis, infestation with liver fluke, and some intestinal parasites.
4. *Certain infections* — scarlet fever, chorea, erythema multiforme, and chronic gonorrhea.
5. *Blood dyscrasias*—Hodgkin's disease, pernicious anemia (especially after raw liver therapy), and after splenectomy.
6. *Following irradiation.*
7. *Familial anomaly.*
8. *Miscellaneous*—periarteritis nodosa, Loeffler's syndrome, tumors of ovary or those involving serous surfaces or bones, and after certain poisons.

D. *Basophilia.*

1. *Infections*—chronic inflammation of sinuses, smallpox, and chickenpox.
2. *Blood dyscrasias*—sometimes in chronic hemolytic anemias, in chlorosis, and following splenectomy.
3. *Miscellaneous*—Hodgkin's disease and following injection of foreign protein.

E. *Lymphocytosis.*

1. *Acute infections*—pertussis, infectious mononucleosis, acute infectious lymphocytosis, mumps, rubella, infectious hepatitis, Dengue fever, and during the stage of convalescence from an acute infection.
2. *Chronic infections*—tuberculosis, brucellosis, congenital and secondary syphilis.
3. Common in bacillary infections.
4. *Miscellaneous*—exophthalmic goiter.
5. Normal in infants and young children.

F. *Monocytosis.*

1. *Bacterial infections* — tuberculosis, subacute bacterial endocarditis, brucellosis, typhus, typhoid fever, and during recovery from acute infections.
2. *Protozoal infections*—malaria, Rocky Mountain spotted fever, kala-azar, trypanosomiasis, oriental sore, and chronic amoebic dysentery.
3. *Miscellaneous*—Hodgkin's disease, Gaucher's disease, carbon tetrachloride poisoning, and during recovery from agranulocytosis.

IV. Leukemia.

A. *Characteristics.*

1. Leukemia is an increase in leukocytes due to an unknown cause; in early cases and in remissions the leukocyte count may be normal or subnormal (aleukemic).
2. There is widespread hyperplasia of the hematopoietic tissue producing the respective cell type.
3. The hyperplasia produces excessive numbers of immature leukocytes which either cir late in the blood stream or become depo in the fixed tissues or both.
4. As a general rule the acute forms have lo leukocyte counts than chronic forms and cur in children or young adults.
5. The chronic forms may simulate the ac forms in the final stage of the disease.
6. The cells are atypical, particularly the mature ones.

B. *Classification* (See Table 15).

1. *Acute Myelocytic Leukemia.*
   a. At least 30 per cent or more of the c are myeloblasts; a few cases have a p ponderance of macromyeloblasts, whil few may have micromyeloblasts.
   b. A few or many promyelocytes present.
   c. Very few mature neutrophilic polym phonuclear leukocytes are present, wh eosinophilic and basophilic forms usually absent.
2. *Chronic Myelocytic Leukemia.*
   a. Very few myeloblasts or promyelocyt
   b. Segmented neutrophils comprise from to 70 per cent of the leukocytes.
   c. Many metamyelocytes and myelocy present.
   d. There is an increase in eosinophilic ar basophilic leukocytes.
3. *Acute Lymphocytic Leukemia.*
   a. May have 50 to 90 per cent lymphoblas
   b. Many prolymphocytes are present.
   c. Only an occasional granulocyte is foun
4. *Chronic Lymphocytic Leukemia.*
   a. From 90 to 99 per cent of the cells a small lymphocytes.
   b. An occasional large lymphocyte with indented nucleus may be found.
   c. A great many smudge cells (degenerat forms) are present.
5. *Acute Monocytic Leukemia.*
   a. At least 20 per cent or more of the cel are monoblasts.
   b. The majority of other cells are immatu monocytes.
6. *Chronic Monocytic Leukemia.*
   a. *Schilling type*—a high percentage, 80 more, of the cells are typical mature mon cytes.
   b. *Naegeli type*—a high percentage of cel called monocytes or monoblasts are prob ably atypical myeloblasts; myelocytes ar usually present.
7. *Rare Leukemias.*
   a. Stem cell—majority of cells are too youn to distinguish the type of cell.

b. Plasma cell leukemia.
c. Chloroma—atypical acute myelocytic leukemia.
d. Eosinophilic leukemia.
e. Basophilic leukemia.
f. Doubtful leukemias — megakaryocytic, lymphosarcoma cell, erythroleukemia, and mixed cell leukemia.

TABLE 15. AVERAGE BLOOD FINDINGS IN DIFFERENT FORMS OF LEUKEMIA

	Acute Myelocytic		Chronic Myelocytic		Acute Lymphocytic		Chronic Lymphocytic		Acute Monocytic		Chronie Monocytic	
	Early*	Late**	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
.B.C. (thousands per c. mm.)	10 -	100	40 -	800	10 -	100	75 -	400	10 -	100	20 -	200
B.C. (mill. per c. mm.)	3 -	1	4 -	2	3 -	1	4 -	2	3 -	1	4 -	2
emoglobin (gm. per 100 cc.)	7 -	3	11 -	6	7 -	3	11 -	6	7 -	3	11 -	6
lor Index	1 -	0.6	1 -	0.8	1 -	0.6	1 -	0.8	1 -	0.6	1 -	0.8
ticulocytes %	5 -	15	1 -	15	3 -	10	1 -	5	2 -	8	1 -	5
atelets (thousands per c. mm.)	100 -	10	200 -	600	100 -	10	150 -	50	100 -	10	300 -	100
fferential Count.												
yeloblasts %	30 -	95	1 -	5								
omyelocytes %	25 -	3	3 -	8								
velocytes												
Neutrophilic %	20 -	2	11 -	28								
Eosinophilic %			6 -	14								
Basophilic %			4 -	9								
etamyelocytes												
Neutrophilic %	10 -	0	10 -	13								
nds												
Neutrophilic %	5 -	0	20 -	8			2 -	0				
gmented												
Neutrophilic %	5 -	0	20 -	6	10 -	0	3 -	0	15 -	0	5 -	0
Eosinophilic %			5 -	3								
Basophilic %			15 -	5								
mphoblasts %					50 -	90						
olymphocytes %					40 -	10	2 -	3				
mphocytes %	5 -	0	3 -	1			90 -	95	10 -	0	15 -	0
onoblasts %									20 -	40	2 -	10
onocytes %			2 -	0			3 -	2	40 -	60	80 -	90
egenerated Forms	Few		Few		Few		Great many		Few		Few	

*Figures under *early* mean average figures for the early stage of the disease.
**Figures under *late* mean average figures for the late stage of the disease.

. *Blood Pictures Resembling Leukemia (Leukemoid Reaction).*

. See Table 16.

. *Diseases with blood picture which may resemble myelocytic leukemia.*
   a. Infections—pneumonia, epidemic meningitis, diphtheria, and tuberculosis.
   b. Intoxications—eclampsia, severe burns, and mercury poisoning.
   c. Malignant tumors with metastasis to bone, multiple myeloma, myelosclerosis, and Hodgkin's disease.
   d. Severe hemorrhage, also sudden hemolysis of blood.

. *Diseases with blood picture which may resemble lymphocytic leukemia.*
   a. Infections—pertussis, chickenpox, infectious mononucleosis.
   b. Malignant tumors with metastasis to bone.

V. Polycythemia.

A. ***Polycythemia*** is an increase above normal in the number of erythrocytes in the circulating blood.

B. ***Relative Polycythemia.***

1. Due to dehydration.
   a. Decrease in fluid intake.
   b. Marked loss of body fluids resulting from persistent diarrhea and vomiting.
2. Hemoconcentration due to shock.
3. Due to some stimulus which releases erythrocytes from the spleen into the circulation.

C. ***Erythrocytosis.***

1. An absolute polycythemia due to a known stimulus.
2. A result of defective saturation of the arterial blood with oxygen, either due to decreased atmospheric pressure or impaired pulmonary ventilation.

3. Due to slowing of the circulation rate (heart disease).
4. Due to a defect in hemoglobin.

D. ***Erythremia (Polycythemia Vera).***

1. An absolute polycythemia of unknown etiology.
2. *Blood Findings.*
    a. Erythrocyte count 7 to 10 million per c. mm.
    b. Hemoglobin 18 to 24 gm. per 100 cc.
    c. Leukocytes range from 6 to 50 thousand per c. mm. with a shift to the left.
    d. Platelets are increased.
    e. Cell pack increased.
    f. The total volume of blood is increased.
    c. Nucleated erythrocytes.
    d. Increased reticulocytes.
    e. Increasing erythrocyte count and hem globin.
    f. Normal indices.
    g. Polychromatophilia.
2. *Signs of increased blood destruction* in an mias due to increased hemolysis.
    a. Increased icterus index.
    b. Increased indirect, but normal direct Va den Bergh test.
    c. Increased urobilinogen in urine and fece
    d. Normal indices.
    e. Poikilocytosis and anisocytosis.
    f. Accompanying signs of increased bon marrow activity.

TABLE 16. DIFFERENTIATION BETWEEN TRUE LEUKEMIA AND LEUKEMOID REACTIONS
(Hill and Duncan)

True Leukemia	Leukemoid Reaction
1. Leukocytes are atypical, particularly the immature ones.	1. Immature as well as mature leukocytes show norma morphology.
2. Myeloblasts may be numerous and as high as 99 per cent.	2. Myeloblasts usually less than 10 per cent.
3. Immature erythrocytes are rarely increased in proportion to the increase of immature leukocytes.	3. Erythroblasts often increased in proportion to immatur leukocytes.
4. Platelets decreased, often severely, except in chronic myelocytic leukemia in which they are increased.	4. Platelets usually normal or increased but may be mod erately decreased.
5. Progressive anemia which may become severe.	5. Anemia variable depending on the cause.

VI. Anemia.

A. ***Anemia*** is a reduction below normal in the number of erythrocytes per c. mm., the quantity of hemoglobin, and the volume of packed erythrocytes per 100 cc. of blood.

B. ***Causes of Anemia.***

1. Increased blood destruction.
    a. Loss of erythrocytes outside the vascular bed—hemorrhage.
    b. Increased destruction of erythrocytes within the vascular bed—hemolysis.
2. Decreased production of erythrocytes or hemoglobin.

C. ***Correlation of Laboratory Findings with Cause of Anemia.***

1. *Signs of increased blood production* in anemias due to blood loss (hemorrhage).
    a. Increased platelets.
    b. Increased leukocytes with a shift to the left.
        1) Normoblasts.
        2) Polychromatophilia.
        3) Increased reticulocytes.
        4) Increased platelets.
        5) Increased leukocytes with a shift t the left.
3. *Signs of decreased production of erythro cytes.*
    a. Decreased platelets.
    b. Decreased leukocytes.
    c. Decreased erythrocytes.
    d. High color index.
    e. Macrocytes.
    f. Megaloblasts.
4. *Signs of decreased production of hemoglobi*
    a. Decreased platelets.
    b. Decreased leukocytes.
    c. Decreased erythrocytes.
    d. Low color index.
    e. Microcytes.
    f. Hypochromia.

VII. Etiological Classification of Anemias.

Anemias mainly due to blood loss or increased blood destruction (bone marrow physiologically hyperactive).

Blood loss.

- a. Acute.
- b. Chronic.*

Increased blood destruction.

- a. *Extrinsic causes.*
  - 1) Infections—streptococcus, Cl. perfringens (welchii), malaria, bartonella, etc.
  - 2) Chemicals—lead, nitro-compounds, etc.
- b. *Intrinsic causes.*
  - 1) Acute hemolytic anemia (Lederer).
  - 2) Paroxysmal hemoglobinuria.
  - 3) Chronic hemolytic jaundice.
  - 4) Sickle cell anemia.

Anemia mainly due to decreased blood production (bone marrow physiologically hypoactive).

Nutritional deficiency of the blood-forming organs.

- a. *Deficiency of the antianemic principle.* (Usually macrocytic hyperchromic anemia.)
  - 1) Pernicious anemia.
  - 2) Pernicious anemia of pregnancy.
  - 3) Macrocytic anemias of gastric resection, intestinal anastomosis, and strictures.
  - 4) Sprue and sprue-like conditions.
  - 5) Macrocytic anemia of pellagra.
  - 6) Macrocytic anemia of liver disease.
  - 7) Parasites (Diphyllobothrium latum).
- b. *Deficiency of iron.* (Usually microcytic hypochromic anemia.)
  - 1) Hypochromic anemias of infancy and childhood.
  - 2) Chlorosis.
  - 3) "Idiopathic" hypochromic anemia.
  - 4) Hypochromic anemia of pregnancy.
  - 5) Hypochromic anemias of gastric resection, intestinal anastomosis, and strictures.
  - 6) Sprue and sprue-like conditions.
  - 7) Parasites (hookworm).
- c. *Deficiency of ascorbic acid.*
- d. *Deficiency of thyroid secretion.*

Toxic inhibition of the blood-forming organs.

- a. *Poisons of external origin:* Benzol, arsphenamine, and gold compounds.
- b. *Toxins of internal origin:* In chronic infections and chronic nitrogen retention.

3. *Physical injury of the blood-forming organs.*
   - a. *External irradiation:* Roentgen ray.
   - b. *Internal irradiation:* Radioactive substances.
4. *Mechanical interference with the blood-forming organs.*
   - a. Acute leukemias.
   - b. Chronic leukemias.
   - c. Multiple myeloma and plasmoma.
   - d. Massive metastatic cancer of the bone marrow.
   - e. Primary xanthomatoses.
   - f. Osteosclerosis.
5. *Idiopathic disturbances of the blood-forming organs.*
   - a. Erythroblastosis fetalis.
   - b. Cooley's anemia.
   - c. "Aplastic" anemia.
   - d. "Splenic" anemia (Banti's syndrome).

VIII. Morphological Classification of the Anemias.

A. ***Macrocytic Anemias.*** (For indices, see Table 7, p. 44.)

1. *Macrocytic hyperchromic anemia* due to deficiency of antianemic principle which is necessary for the maturation of the erythrocytes from the megaloblastic stage—pernicious anemia and anemia of liver disease and pellagra.
2. *Macrocytic normochromic anemia* due to acute blood loss or increased blood destruction if accompanied by markedly hyperactive bone marrow.
3. *Macrocytic hypochromic anemia* found in:
   - a. Pernicious anemia of pregnancy.
   - b. Macrocytic anemia of gastric resection, intestinal anastomosis, and strictures.
   - c. Sprue and sprue-like conditions.
   - d. Parasites (Diphyllobothrium latum).

B. ***Normocytic Normochromic Anemia***—May be due to one of the following causes (for indices, see Table 7):

1. *Acute blood loss*—posthemorrhagic anemias.
2. *Increased blood destruction*—hemolytic anemias.
3. *Decreased blood production* resulting from reduced function of the erythropoietic tissue, due to the following:
   - a. *Inhibiting effect of toxins or poisons* as in infections or chronic kidney disease.
   - b. *Injury by physical agents,* such as Roentgen ray or radium.
   - c. *Mechanical interference* as in replacement of bone marrow by tumor, leukemic tis-

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*Anemia due to chronic blood loss differs from the other types in this group; physiologically it belongs with hypochromic anemias due to deficiency of iron. The bone marrow is physiologically hypoactive.

sue, or by overgrowth of bone (generalized osteosclerosis).

   d. *Idiopathic disturbances*—congenital anemias of infants and erythroblastic, aplastic, and splenic anemias.

C. *Microcytic Anemias.* (For indices, see Table 7 on page 44.)

1. *Microcytic normochromic anemia* due to imperfect blood formation.
   a. Subacute and chronic inflammations, such as nephritis.
   b. Chronic noninflammatory diseases, such as malignant tumors.
2. *Microcytic hypochromic anemia* due to deficiency of iron.
   a. Iron is necessary for the completio maturation from the normoblastic s and the normal filling of erythrocytes hemoglobin.
   b. Deficiency of iron may be due to the lowing:
      1) Inadequate intake.
      2) Faulty absorption.
      3) Defective storage in liver.
      4) Continued loss of blood.
      5) Excessive demands.

D. *Laboratory Findings in Anemias.* Tables 17 and 7.

Table 17. Average Findings in Different Forms of Anemia

	Normal	Microcytic Hypochromic Anemia	Macrocytic Hyperchromic (Pernicious) Anemia	Anemia in Chronic Hemolytic Jaundice**
Hemoglobin (gm. per 100 cc.)	14 - 16	4 - 8	5 - 8	5 - 10
R.B.C. (mill. per c. mm.)	4.5 - 6	3 - 4.5	1 - 2.5	1.5 - 4
W.B.C. (thousands per c. mm.)	5 - 10	5 - 7	3 - 4	7 - 10
Color Index	0.9 - 1.1	0.4 - 0.8	1.2 - 2	0.8 - 1
Mean Corpuscular Hgb. (micromicrograms)	27 - 32	14 - 21	30 - 52	27 - 32
Volume Index	0.9 - 1.1	0.7 - 0.9	1.2 - 2	0.7 - 1
Mean Corpuscular Volume (c. microns)	80 - 94	50 - 71	95 - 160	77 - 87
Saturation Index	1.0	0.6 - 0.9	0.9 - 1.0	1.0
Mean Corpuscular Hgb. Conc. (%)	32 - 36	21 - 29	31 - 36	32 - 36
Macrocytosis	None	None	++ to +++	None
Microcytosis	None	++ to +++	+	++
Hypochromia	None	++ to +++	None	None
Megaloblasts	None	None	+	+
Normoblasts	None	+	+	++ to ++
Reticulocytes (%)	0.5 - 1.5	1 - 3	1 - 3*	10 - 40
Platelets (thousands per c. mm.)	200 - 400	250 - 300	50 - 150	300 - 400
Icterus Index	4 - 6	3 - 5	10 - 30	10 - 50
Fragility range	0.30 - 0.44	Normal	Normal	0.48 - 0.8
Indirect Van den Bergh	Normal	Normal	+	+
Urobilinogen (urine)	Normal	Normal	+	+

+Slight. ++Moderate. +++Marked.

*Great increase after liver therapy.

**Hereditary factor, usually splenomegaly.

Gastric and Duodenal Contents

General Considerations

Digestion in the stomach consists mainly of the tion of pepsin upon proteins in the presence of drochloric acid and in the curdling of milk by nnin. The gastric mucosa secretes pepsinogen, nninogen, and hydrochloric acid, the latter nverts the former two into the enzymes, pep- and rennin. The hydrochloric acid also com- nes loosely with the proteins of the food rming acid metaprotein, the first step in pro- in digestion. This acid metaprotein is called ombined" hydrochloric acid. The acid secreted ter the proteins are converted to acid metapro- in remains as "free" hydrochloric acid. This ee acid continues the process of digestion, acts an antiseptic on ingested bacteria, and pro- otes the absorption of iron by transforming rric to ferrous salts. The amount of acid in e stomach is an indicator of the secretory ac- ity of the stomach. In addition to the produc- n of acid and enzymes, the stomach secretes the ntrinsic" factor which with the "extrinsic" etary factor (B_{12}) produces the erythrocyte turation factor.

Normally the stomach secretes gastric juice en stimulated by the presence of food; there- e, gastric analysis is usually made after a test al or histamine injection, but it may also be de on the fasting contents removed from the mach 12 or more hours after a meal. The fol- ving are contraindications of passage of a mach tube: stenosis or malignant tumor of esophagus, aortic aneurism, esophageal vari- or diverticula, recent severe gastric hemor- ge, and cardiac decompensation. In preg- t or severely ill persons, a tube should be sed only upon urgent indication.

Obtaining Contents

I. Withdrawal of Contents.

Types of Stomach Tubes.

An Ewald or Boas tube is a flexible rubber tube about 12 mm. in diameter.
   a. A rubber bulb is used as the aspirator.
   b. This type of tube is used when only one specimen is desired or for washing out the stomach in cases of poisoning.

The Levine tube is a soft rubber catheter with several openings near the tip.
   a. It is marked at 50, 60, 70, and 80 cm.
      1) The first mark indicates the distance from the lips to the cardiac end of the stomach.
      2) The third mark indicates the distance to the pylorus.
      3) The fourth mark indicates the distance to the duodenum.
   b. It is used when the tube is left in the stomach for a period of time as in the fractional test.
   c. The gastric contents are aspirated with a glass syringe.

3. The Rehfuss tube is similar to the Levine tube except it has a metal tip on one end and is usually smaller in diameter. It is used mainly for duodenal or biliary drainage.

B. *Passing of Stomach Tube.*

1. Cover the patient's clothing with towels or an apron and give him a pan to hold. Assure him that the introduction of the tube can do him no harm and that if he can control the spasm in his throat, he will experience very little choking sensation.
2. The tube should be cold. Let it stand in cold water 30 minutes before using.
3. The patient should be instructed to breathe through his mouth.
4. His head should be held down with the mouth open and the tongue out forming a groove.
5. The tip of the tube is introduced far back into the pharynx.
6. The patient is urged to swallow and the tube is pushed into the stomach until the second mark on the tube reaches the incisor teeth. If it is impossible for the patient to swallow the tube, the throat may be sprayed with 1% cocaine.
7. The patient should expectorate into the pan any saliva which appears in the mouth, for if swallowed it interferes with the test.
8. A Levine tube may be introduced through the nose.
   a. Lubricate the tip with a few drops of glycerin or vaseline.
   b. Introduce the tube along the floor of the nose until the tip turns down into the pharynx.

   c. Have the patient swallow repeatedly and push the tube down slowly as he swallows until it is well into the esophagus, then it may be rapidly passed to the third mark.

C. ***Withdrawing Contents.***

1. When an Ewald tube is used, pass it into the stomach, compress the bulb, attach it to the end of the tube, and withdraw the entire contents.
   a. When no more contents can be obtained, pull the tube out about an inch and try again; repeat this until the first mark on the tube is at the incisor teeth.
   b. Push down to the second mark again and see if any more contents can be obtained.
   c. Pinch the tube to retain any contents and pull it out.
2. In the fractional test with the Rehfuss or Levine tube, samples are removed at definite intervals with a glass syringe.
3. After removing the tube at the end of the test, examine the tip for mucus, blood clots, tissue fragments, and food particles.
4. Clean the tubes, glass syringe, and bulb with running water. Sterilize by boiling in distilled water for 5 minutes and then fold in clean towels ready for use.

II. Fasting Contents.

A. ***Instructions to Patient.***

1. Eat a meat sandwich, 20 raisins, and drink one glass of water before retiring the night before reporting to the laboratory.
2. Do not eat or drink anything after this meal until the stomach contents are removed.
3. Do not brush teeth the morning of the test as swallowed blood from bleeding gums will give a positive blood test.

B. ***Obtaining Contents.***

1. Twelve hours after the above meal pass an Ewald or Levine tube as described under passing stomach tube.
2. Remove the fasting contents.
3. See examination of fasting contents on page 103.

III. Gastric Washings for Tubercle Bacilli.

A. ***Obtaining Material.***

1. Should be done as soon as possible after the patient awakens in the morning.
2. No food or *fluid* should be taken.
3. Pass a Rehfuss or Levine tube as described under passing stomach tube.
4. Pump out the fasting contents and place in a sterile 50 cc. centrifuge tube.
5. Inject 50 cc. of warm 0.85% NaCl solution through the tube into the stomach.
6. Wash out the stomach by withdrawing ar reinjecting the saline solution 3 or 4 tim
7. Withdraw contents and place in anoth sterile 50 cc. centrifuge tube.
8. If the fasting contents and washings cannot b immediately digested for the guinea pig i oculation, they should be pooled and th neutralized with a 2% solution of NaOH.

B. ***Guinea Pig Inoculation.*** See directions page 200.

IV. Duodenal Drainage.

A. ***Passing Tube into Duodenum.***

1. Patient must not have had any food for 1 hours.
2. Pass a Rehfuss tube with a metal tip int the stomach to the second mark as describe under passing stomach tube.
3. Remove any material in the stomach an wash stomach repeatedly with warm wate until it returns clear.
4. Place patient on right side, elevate the hip 6 to 8 inches, and flex the right knee.
5. This position permits gravity to aid peristalsis in carrying the end of the tube throug the pylorus, also in letting the gastric an duodenal contents drain spontaneousl through the tube.
6. Push the tube in slowly—1 inch a minut to the third mark.
7. Place the end of the gastric tube in a larg test tube.
8. Replace test tube with a clean tube every 3 minutes.
9. Test the reaction of the contents of the tube when alkaline the tube has passed into th duodenum.
10. A fluoroscopic examination may be necessary to verify the position of the tube i the duodenum.

B. ***Collection of Duodenal Contents.***

1. Collect the duodenal contents in a clean tes tube.
2. The contents are generally less than 10 cc. slightly syrupy, water clear, or pale yellow.

V. Biliary Drainage.

A. ***Stimulation of Secretion.***

1. Pass a Rehfuss tube into the duodenum (se duodenal drainage).
2. Inject 3 portions of 25 cc. each of warm sterile 25% magnesium sulfate solution into the tube at 10 minute intervals.
3. The magnesium sulfate relaxes the sphincter of Oddi and stimulates the flow of bile.

The bile drains spontaneously, gradually changing from one type to the next.

Types of Bile.

The first bile, "A," to drain is golden yellow color and comes from the common bile duct. Collect in a test tube labeled A. The amount varies from 5 to 30 cc.

The second type of bile, "B," is yellow-brown to olive green, viscid, and comes from the gallbladder. Collect in another test tube labeled B. The amount varies from 30 to 60 cc. The terminal portions are used for bacteriological study.

The third type of bile, "C," is light yellow and comes from the liver. Collect in a test tube labeled C. The amount varies from 30 to 200 cc.

Different Types of Tests

I. Ewald Test Meal (Modified).

Routine Method.

The patient is instructed to take no fluid or food after the evening meal of the day preceding the test.

On the following morning an Ewald test meal consisting of six Arrowroot cookies and 2 glasses of water (about 300 to 400 cc.) is given to the patient.

The contents of the stomach are removed after 50 minutes, counting from the beginning, not the end, of the meal.

Make a routine examination of the contents.

Fractional Method.

Give a meal as in the routine method.

At 30 minute intervals for 2 hours, remove samples of about 15 cc. each. At the end of 2 hours remove everything remaining in the stomach.

Measure the amount in each of the 30 minute specimens, filter, and titrate the free hydrochloric acid and total acid in each.

Do a complete routine examination on the specimen removed at the end of the first hour.

If there is no free hydrochloric acid in any of the specimens taken during the first hour, inject 0.25 mg. of histamine dihydrochloride or acid phosphate (0.25 cc. of a 1:1000 dilution) subcutaneously and continue test as described above.

II. Alcohol Test Meal

Obtaining Contents.

The patient is instructed to take no food or fluid after the evening meal of the day preceding the test.

2. On the following morning pass a Levine tube and withdraw all the fasting contents. Leave the tube in place.
3. Inject 50 cc. of 7% alcohol through the tube into the stomach.
4. Withdraw all the contents obtainable every 30 minutes for 2 hours and place each in a separate test tube.

B. ***Examination of Contents.***

1. Measure the amount and titrate the free hydrochloric acid and total acid of each specimen including the fasting contents.
2. Run a complete analysis on the first hour specimen.

III. Histamine Test.

A. ***Obtaining Contents.***

1. The patient is instructed to take no food or fluid after the evening meal of the preceding day.
2. On the following morning pass a Levine tube and withdraw all the fasting contents. Leave the tube in place. The patient should be advised to take particular care not to swallow saliva as histamine stimulates the salivary glands.
3. *Amount of Histamine.*
   - a. Inject 0.25 mg. of histamine dihydrochloride or acid phosphate (0.25 cc. of a 1-1000 dilution) subcutaneously, care being taken not to get any in a vein.
   - b. A dose of 0.1 mg. for each 10 kilograms of body weight is preferred by some, but it may produce a reaction (flushing of face, quickening of the pulse, and physical discomfort).
   - c. Contraindications to histamine are low blood pressure or an unstable vasomotor system.
4. Withdraw all the contents obtainable at 20 minute intervals for 80 minutes and place each in a separate test tube.

B. ***Examination of Contents.***

1. Measure the amount and titrate the free hydrochloric acid and total acid of each specimen including the fasting contents.
2. Run a complete analysis on the 40 minute specimen.

Routine Examination of Gastric Contents

I. Physical Examination.

A. ***Quantity.***

1. Normally there are from 20 to 100 cc. of gastric contents after an Ewald meal. From 15 to 50 cc. are obtained 20 minutes after a histamine injection.

2. An increase may occur in obstruction at or near the pylorus and in acute or chronic dilatation of the stomach.
3. A decrease may be due to hyposecretion or hypermotility with too rapid emptying of the stomach. Incomplete removal of the contents must be ruled out.

B. *General Appearance.*

1. Normal gastric contents are watery with a mixture of food particles, which should be very fine after an hour's digestion and which settle to the bottom upon standing.
2. The proportion of solid to liquid should be about 1 to 3.

C. *Color.*

1. *Normally* the solid portion is opaque gray; the supernatant fluid is slightly cloudy.
2. *Abnormally* it is colored by:
   a. *Different kinds of food* left in the stomach because of stasis.
   b. *Blood.*
      1) Red if fresh. If in clumps, it is probably due to trauma from swallowing the tube and should be so reported.
      2) Coffee ground color after it has been acted upon by gastric juice.
   c. *Bile.*
      1) Yellow if fresh, greenish if old.
      2) Fresh bile is sometimes normally present due to excessive straining.
      3) If present in repeated tests, is usually due to some disease.
   d. *Feces*—brownish black in intestinal obstruction.

D. *Odor.*

1. Normally odorless or slightly sour.
2. Alcoholic in alcoholic coma and after an alcohol test meal.
3. Ammoniacal in uremia.
4. Fecal in intestinal obstruction.
5. Rancid in benign stenosis and fermentation.
6. Putrid odor in pyloric stenosis due to ulcerated necrotizing carcinoma.

E. *Mucus.*

1. Swallowed mucus is in masses.
2. If from the stomach, it is intimately mixed with the contents, is slimy in appearance, and adheres to the side of the glass container when it is tipped.
3. If present in large quantities, it suggests a catarrhal type of inflammation.

II. Chemical Examination.

A. *Reaction.*

1. Test with Congo red paper; if free hydrochloric acid is present, the paper will turn blue.
2. The pH is an index of activity of the acid rather than the amount.
   a. A pH of 1.6 to 1.8 is normal.
   b. A pH of 1.4 or lower represents a
   acidity.
   c. A pH of 2.0 to 2.8, a low acidity.
   d. A pH of 3.0 or higher, achlorhydria
   acidity).
   e. A pH of 7, true anacidity, is a rare
   dition.
3. May be neutral or alkaline due to con
nation with an excess of saliva, ingestio
alkaline material, or regurgitation of bile
duodenal fluid.

B. *Acids.*

1. *Free Hydrochloric Acid (HCl).*
   a. Filter enough gastric contents thr
   gauze so that 10 cc. can be measured
   a pipette into a 100 cc. beaker or f
   (When volume is very small, centrifu
   b. Add 4 drops of 0.5% alcoholic solu
   of p-dimethylaminoazobenzene (Töp
   reagent).
   c. A red color appears if free HCl is pres
   d. Add 0.1 N NaOH from a burette u
   the initial red color has been repla
   by a salmon pink color (save for ti
   tion of combined acid).
   e. The number of cc. of 0.1 N NaOH u
   multiplied by 10 gives the amount of
   HCl present in 100 cc. of contents,
   pressed in terms of cc. of 0.1 N Na
   f. Acidity may also be expressed in degr
   each cc. of 0.1 N NaOH per 100 cc.
   gastric contents represents one degree
   acidity.
   g. If it is desired to express the acidity in
   cent of HCl; that is, the amount of
   by weight in 100 cc. of gastric juice, m
   tiply the value obtained in (e) by 0.00
   since 1 cc. of 0.1 N HCl contains 0.00
   gm. HCl.
   h. *Normal values (euchlorhydria).*
      1) Fasting—5 to 20 degrees, howeve
      per cent of children and 30 per c
      of adults have no free HCl.
      2) Fifty minutes after an Ewald mea
      20 to 60 degrees.
      3) One hour after an alcohol meal—
      to 70 degrees.
      4) One hour after histamine—30 to
      degrees.
   i. *Abnormal values.*
      1) Increased (hyperchlorhydria) in n
      rosis, gastric ulcer, and beginn
      chronic gastritis.
      2) Decreased (hypochlorhydria)
      chronic gastritis, early gastric car
      noma, pellagra, and in some neuros

    3) Absent (achlorhydria) in gastric carcinoma, syphilis of the stomach, chronic atrophic gastritis, pernicious anemia, hypochromic microcytic anemia, chronic alcoholism, sprue, many cases of pellagra, and sometimes in hysteria and pulmonary tuberculosis.
    4) Achylia gastrica (complete absence of free HCl and ferments) in chronic atrophic gastritis, pernicious anemia, gastric carcinoma, and hypochromic microcytic anemia.

Combined Acid.

a. Add 4 drops of 1% alcoholic solution of phenolphthalein to the same sample of gastric contents previously used for titrating free HCl.
b. Add 0.1 N NaOH until the first permanent pink (not red) color appears.
c. The number of cc. used in this second titration multiplied by 10 equals the amount of combined acid in 100 cc. of contents, expressed in cc. of 0.1 N NaOH or as degrees.
d. *Normal values.*
    1) Fasting—10 to 15 degrees.
    2) Fifty minutes after an Ewald meal—20 to 30 degrees.
e. *Abnormal values* (decreased) are found when there is an achlorhydria.

Total Acid.

a. The free HCl plus the combined acid equals the total acidity.
b. Total acidity includes free HCl, combined HCl, organic acids, and acid phosphates.
c. *Normal values.*
    1) Fasting—15 to 45 degrees.
    2) Fifty minutes after an Ewald meal—50 to 75 degrees.
d. *Abnormal values* found in same conditions as described under free HCl.
    1) Increased in cases with hyperchlorhydria.
    2) Decreased in cases with hypochlorhydria and achlorhydria.

Lactic Acid (Kelling's Test).

a. *Principle:* Ferric chloride is converted to ferric lactate by lactic acid.
b. Always test for lactic acid if the free HCl is below 20 degrees.
c. To a test tube of water, add 3 to 4 drops of 10% ferric chloride solution.
d. Shake the tube and pour one half of the contents into another test tube of the same size for a control.
e. Add 1 cc. of filtered gastric contents to one tube and if lactic acid is present a distinct brilliant yellow color will immediately appear.
f. Normally it is not present at the height of digestion.
g. It may be present in the following:
    1) Whenever there is a decrease or absence of free HCl due to carcinoma, dilatation of the stomach, or chronic gastritis.
    2) In stagnation due to pyloric obstruction associated with hypochlorhydria.

C. *Pepsin and Pepsinogen.*

1. Preparation of egg albumin disks.
    a. Boil an egg very slowly until the albumin is coagulated.
    b. Remove the shell and yolk.
    c. With a cork borer cut the coagulated albumin (egg white) into small cylinders about 5 mm. in diameter.
    d. Section the cylinders into disks about 1 mm. thick.
    e. The disks may be preserved in glycerin until needed, but must be washed in water before being used.
2. Place an albumin disk into each of 3 test tubes marked 1, 2, and 3.
3. In tube 1 place 10 cc. of distilled water, 5 grains of pepsin, U.S.P., and 3 drops of 10% HCl.
4. In tube 2 place 10 cc. of filtered gastric juice.
5. In tube 3 place 10 cc. of filtered gastric juice and 3 drops of 10% HCl.
6. Place the tubes in a 37°C. incubator or water bath for 3 hours or longer and observe at 30 minute intervals.
7. *Results.*
    a. Tube 1 is used for comparison and should show the effect of normal gastric juice, the albumin should be completely digested in 3 hours.
    b. Digestion of the albumin in tube 2 indicates the presence of both pepsin and free HCl in the gastric juice.
    c. No digestion in tube 2 and digestion in tube 3 indicates pepsinogen is present, having been converted to pepsin by the HCl added.
    d. If digestion fails to take place in tube 3, both pepsin and pepsinogen are absent.

D. *Rennin.*

1. Neutralize 5 cc. of gastric juice with 0.01 N NaOH using phenolphthalein as an indicator.
2. Add 5 cc. of fresh milk and place in a 40°C. water bath.
3. A normal amount of rennin will coagulate the milk in 10 to 15 minutes.
4. Delayed coagulation indicates a diminished amount.

E. *Blood.*

1. Normally no blood is found; small clumps may be present due to trauma in passing the tube.
2. Found in peptic ulcers, gastric carcinoma, varices of the esophagus or stomach, chronic passive congestion, violent or prolonged vomiting, bleeding papilloma, or blood dyscrasia.
3. Vomiting of blood (hematemesis) may be mistaken for pulmonary hemorrhage (hemoptysis).
   a. In hematemesis the fluid is acid in reaction, usually dark red or brown in color, and clotted.
   b. In hemoptysis the fluid is brighter red, frothy, alkaline, and usually mixed with mucus.
4. See page 14 for principle and general consideration of the blood tests.
5. *Benzidine Test.*
   a. Place a pocket-knife point full of benzidine in a test tube.
   b. Add 3 cc. of glacial acetic acid and shake until the acetic acid is thoroughly saturated. If necessary, add more benzidine.
   c. Allow the benzidine to settle and pour the clear supernatant liquid into another test tube. (A saturated solution, 4 gm. of benzidine in 100 cc. of glacial acetic acid, will keep for 2 weeks in a brown bottle.)
   d. To this fluid add 2 cc. of unfiltered gastric contents and mix. (If there is fat in the gastric contents, see confirmatory test below.)
   e. Add 1 cc. of fresh hydrogen peroxide and mix.
   f. A blue or green color appearing within 5 minutes indicates the presence of blood.
   g. If positive, report as follows:

      Trace = faint green
      + = green
      + + = greenish blue
      + + + = blue
      + + + + = deep blue

   h. This test is more sensitive than the guaiac test.
6. *Guaiac Test.*
   a. Place a pocket-knife point full of powdered guaiac in a test tube, add 2 cc. of 95% alcohol, and mix. (A 1:25 solution of guaiac in alcohol keeps for 8 months in a brown bottle.)
   b. Add 2 cc. of fresh hydrogen peroxide and shake.
   c. To 4 cc. of gastric contents (not filtered), add 3 or 4 drops of glacial acetic acid and mix thoroughly. (If there is fat the gastric contents, see confirmatory tes
   d. Pour the guaiac solution slowly down t side of the tube to form a layer on t of the gastric contents.
   e. If blood is present, a green to blue co will appear at the zone of contact.
   f. The intensity of color and width of t zone will vary with the amount of blo present. See benzidine test for method reporting results.
7. *Confirmatory Test.*
   a. If fat is present, make 10 cc. of unfiltere gastric contents slightly alkaline to litm or nitrazine paper with a few drops 10% NaOH.
   b. Extract with an equal amount of ethe shaking gently, then discard the eth which contains the fat.
   c. Make the gastric contents strongly ac with a few drops of glacial acetic aci and extract again with ether, which wi contain any hemoglobin present.
   d. Place the ether in an evaporating dish an evaporate to dryness over a water bat
   e. Dissolve the residue in 1 cc. of water, the add either benzidine or guaiac as describe in above methods.

F. *Bile Pigment.*

1. Shake 5 or 10 cc. of unfiltered gastric con tents with an equal amount of 10% bariu chloride solution and let stand a few minute
2. Filter through a small filter paper.
3. Partially dry the filter paper and add a dro of yellow nitric acid or Fouchet's reagent t the center of the precipitate on the filte paper.
4. If positive with nitric acid, there is a displa of colors, green on the periphery, then i order toward the center, blue, violet, red, an yellow. The absence of green excludes th presence of bile.
5. If positive with Fouchet's reagent, there is blue to green color.
6. For principle of test and its reagents, see pag 12 in Section on Urinalysis.
7. Small quantities of fresh yellow bile may b normally present.
8. Large quantities of old bile (green) in pa tients accustomed to the stomach tube ma indicate duodenal obstruction, hyperchlorhydria, biliary tract disease, duodenal ulcer, o a rigid pylorus due to scarring or adhesions

III. Microscopic Examination.

A. ***Smear***—make a smear of the unfiltered con tents, dry, and stain by Gram's method.

Examine for the following:

Pus Cells—normally not present but may be found in a few cases of gastric carcinoma or gastritis.

Erythrocytes—when only a few corpuscles are present, they are usually due to irritation of the gastric mucosa by the tube.

Mucosa—occasionally small fragments are found in some cases of carcinoma, hypertropic gastritis, atrophic gastritis, or ulcers.

Epithelial Cells—squamous cells derived from the esophagus have no significance. Columnar cells may be found in gastritis. Deeply bile-stained columnar cells in fasting contents may indicate gallbladder disease.

Yeast Cells—a few may be found normally, but a large number indicate retention and fermentation.

Bacteria—numerous bacteria may be present especially in absence of free HCl. The predominant organisms are gram-positive. Only the following have significance.

a. *Sarcinae* are large gram-positive cocci, arranged in cuboid groups. They are only significant in large numbers and indicate stasis without decrease in acidity. They are found in ulcers producing pyloric obstruction. Their presence usually excludes carcinoma.
b. *Boas-Oppler* bacilli are large (5 to 10 microns long) gram-positive bacilli arranged in clumps or end to end in zigzag chains. They are important only when found in large numbers. They occur in the majority of cases of carcinoma, especially those causing pyloric obstruction, and indicate achlorhydria with gastric stagnation.

Examination of Fasting Contents

I. Quantity.

Normal Amount—usually 10 to 50 cc.

Abnormal Amount—over 50 cc. indicates hypersecretion, hypomotility, or obstruction.

II. General Appearance.

Normal Contents.

Gray, thin mucoid fluid wtih 5 per cent sediment consisting of exfoliated epithelium, few leukocytes, and a few bacteria.

Bile may normally be present in 2 conditions:

a. Reversed peristalsis if the patient gags in swallowing the tube.
b. Regurgitation of duodenal contents in a physiological attempt to lower a hyperacidity.

B. *Abnormal Contents.*

1. May be red from fresh blood, or dark "coffee grounds" from altered blood.
2. Cloudy from food, pus, or epithelium.
3. Syrupy or ropy from an excess of mucin.
4. Bile colored.
5. Macroscopic food always indicates stasis.

III. Mucus.

A. *Normally* present in stomach contents in small quantities.

B. *Abnormal Amounts.*

1. When sufficient to give a slight white cloudy precipitate on the addition of a drop of glacial acetic acid to the filtered contents (too much acetic acid dissolves mucus).
2. Marked excess of mucus is significant of catarrhal gastritis, achylia, and in great quantity of gastromyxorrhea.

IV. Chemical Tests.

See routine examination of gastric contents, page 100.

V. Microscopic Examination.

A. *Unstained Smear.*

1. Look for meat fibers, striated or partially digested, and fruit pulp.
2. Starch granules stain blue with a drop of Lugol's solution.
3. Fat globules stain orange with Sudan III.
4. Normally there should be no food particles present 5 hours after a meal.

B. *Stained Smear (Gram Stain).*

1. Excessive exfoliation of epithelium means local inflammatory changes.
2. Abnormal increase in pus cells or their nuclear remains suggests inflammation.
3. Excessive bacterial flora, especially if in colony formation, suggest infection.
    a. Sarcinae and many yeast cells suggest gastric dilatation with stagnation and fermentation of contents.
    b. Boas-Oppler bacilli found in subacid or anacid gastric juice are associated with retention and stagnation.

Examination of Duodenal Contents

I. Macroscopic Examination.

A. *Physical Characteristics.*

1. Normally water clear, faintly cloudy, or pale yellow.
2. Marked opacity may be due to admixture with gastric contents or to local inflammation.

B. *Blood.*

1. Normal duodenal contents or fresh bile may give a positive benzidine test.
2. A large amount of blood may indicate an ulcer or carcinoma.

II. Chemical Tests.

A. *Trypsin.*

1. *Solutions.*
   a. *Phosphate buffer solution of pH 8.4*—see page 260.
   b. *Casein solution.*
      1) Place exactly 1 gm. of soluble casein in a flask.
      2) Add 100 cc. of phosphate buffer solution and dissolve the casein by quickly rotating the flask.
      3) The solution may be carefully warmed to 57°C. but care must be taken not to obtain a doughy mass.
      4) When the casein is dissolved, neutralize with 4 cc. of 0.1 N NaOH.
      5) This solution must be made fresh each day.
   c. *Metaphosphoric acid solution* (25%)—freshly prepared.
   d. Solutions for nonprotein nitrogen determination, see page 274.
2. *Method.*
   a. Centrifuge the duodenal contents until clear.
   b. Place 1 cc. of the clear supernatant fluid in a 50 cc. volumetric flask and make up to the mark with phosphate buffer solution.
   c. Place 9 cc. of casein solution in each of 2 large test tubes (100 x 10 mm.) labelled 1 and 2, and heat in a water bath at 40°C. for 5 minutes.
   d. To tube 1 add 1 cc. of the diluted duodenal contents and mix.
   e. To tube 2 (blank) add 1 cc. of the diluted duodenal contents which has been heated in a water bath of boiling water for 10 minutes to destroy the activity of the enzyme.
   f. Incubate both tubes for 30 minutes in a 40°C. water bath.
   g. Add 2 cc. of freshly prepared 25% metaphosphoric acid to each tube, mix thoroughly, and filter. The filtrate should be clear and colorless.
   h. Place 1 cc. of the corresponding filtrate and 1 cc. of digestion mixture in pyrex test tubes numbered 1 and 2 and continue as described under nonprotein nitrogen determination on page 272.
   i. *Calculation:*

$$\frac{RS}{RU} \times \frac{0.15 \times 600 \times 100}{1000} = \frac{180}{RU} = \text{gm.\% of nitro}$$

   Subtract gm. per cent of nitrogen in tu 2 (blank) from tube 1 to obtain th activity of trypsin in 100 cc. of duoden contents.
   j. The photoelectric colorimeter method fo nonprotein nitrogen may be used; how ever, the mg. of nitrogen in the sample an the dilution of the duodenal contents mu be used in the calculation and the resu reported in grams per cent.
3. *Interpretation of Trypsin Findings:*
   a. Normal values: 7.5—15 gm. of nitroge per 100 cc. of duodenal contents.
   b. Decreased in:
   Same as for *Amylase.* See page 105.

B. *Amylase* (Amylopsin).

1. *Solutions.*
   a. *Phosphate buffer solution of pH 8.4*—se page 260.
   b. *Starch phosphate mixture.*
      1) Completely dissolve 4 gm. of solubl starch in 100 cc. of hot distilled water
      2) Cool and dilute with 100 cc. of phosphate buffer solution.
   c. Solutions for Folin and Wu's blood suga method, see page 266.
2. *Method.*
   a. Centrifuge the duodenal contents unti clear.
   b. Place 1 cc. of the clear supernatant fluid in a 25 cc. volumetric flask and dilute to the mark with phosphate buffer solution.
   c. Place 9 cc. of starch phosphate mixture in each of 2 test tubes labeled 1 and 2, and heat in a water bath at 40°C. for 5 minutes.
   d. To tube 1 add 1 cc. of the diluted duodenal contents and mix.
   e. To tube 2 (blank) add 1 cc. of the diluted duodenal contents which has been heated in a water bath of boiling water for 1 minutes to destroy the activity of the enzyme.
   f. Incubate both tubes for 30 minutes in the 40°C. water bath.
   g. Place 2 cc. of alkaline copper tartrat solution (Folin and Wu's) in each of blood sugar tubes labeled 1 and 2 and add 2 cc. of incubated starch solution from the tube with the corresponding number.
   h. Rotate and place the tubes in boiling wate for 6 minutes.
   i. Cool in a cold water bath for 3 minutes then add 2 cc. of molybdate solution t each.

j. Dilute to mark with distilled water, mix, and read in the colorimeter against the 0.1 mg. standard run at the same time as the duodenal contents. Set standard at 20.

k. *Calculation:*

$$\frac{RS}{RU} \times \frac{0.2 \times 250 \times 100}{1000 \times 2} = \frac{50}{RU} = \text{gm.\% of glucose.}$$

Subtract gm. per cent of glucose in tube 2 (blank) from tube 1 to obtain the amylase in 100 cc. of duodenal contents.

l. The photoelectric colorimeter method for blood sugar may be used, but the proper dilutions must be taken into account for the calculation and the result reported in grams per cent.

Interpretation of Amylase Findings:

a. Normal value for glucose liberated in 30 minutes is 2.5 to 5 gm. per 100 cc. of duodenal contents.

b. Decreased in:
   1) Pancreatic insufficiency and chronic pancreatitis.
   2) Slight decrease in celiac disease.
   3) Absent in fibrocystic disease of the pancreas.

III. Microscopic Examination.

. ***Preparation.***

. The specimen must be examined at once before the ferments destroy the cellular elements.

. Centrifuge contents and pour off the supernatant fluid.

. Make a wet preparation by placing a drop of the sediment on a slide, cover with a cover glass, and examine with the low and high power objectives.

. Make a smear of the sediment, dry, and stain by Gram's method.

. ***Findings.***

. *Wet Preparation:*

a. A few epithelial and pus cells are normally found; a marked increase suggests inflammation.

b. Mucus is usually present but has no significance.

c. The following parasites may be found:
   1) Strongyloides stercoralis, see Fig. 14, page 130.
   2) Giardia lamblia, see Fig. 17, page 143.
   3) Cystic and vegetative forms of Endamoeba histolytica, see Fig. 17, page 143.
   4) Ova of Necator americanus, see Fig. 14, page 130, and Clonorchis sinensis, see Fig. 16, page 138.

2. *Stained Smear*—identify types of cells present and bacteria.

Examination of Bile

I. Macroscopic Examination.

A. ***Color.***

1. See biliary drainage, page 98.
2. Failure to obtain "B" bile suggests that the gall bladder is not storing or concentrating bile.
3. Dark bile may originate from a dilated common duct when the gall bladder is obliterated or has been removed, but "A" bile would also be dark.

B. ***Report*** degree (+ to ++++) of viscosity and turbidity of each type of bile.

II. Microscopic Examination.

A. ***Preparation.***

1. Centrifuge each specimen separately at a high rate of speed and pour off the supernatant fluid.
2. Make a wet preparation by placing a drop of sediment on a slide, cover with a cover glass, and examine with the low and high power objectives of the microscope.
3. Make a smear of the sediment, dry, and stain by Gram's method.

B. ***Findings.***

1. *Wet Preparation.*
   a. An occasional bile stained epithelial or pus cell may be found; a marked increase suggests inflammation.
   b. Cholesterol appears as flat, colorless, thin crystals with chipped edges and usually means formed or forming calculi.
   c. Bilirubin appears as amber, brown, or black amorphous material; calcium bilirubinate as clusters of lustrous bright yellow, lemon or orange, fine or coarse granules. These have same significance as cholesterol.
2. *Stained Smear*—identify types of cells present and bacteria.

III. Bacteriological Examination.

A. ***Collection.***

1. Must be collected under aseptic conditions.
2. Excessive gagging or retching causes contamination.

B. ***Examination.***

1. Centrifuge each specimen separately, make smears of the sediment, and stain by Gram's method.
2. Make cultures of the sediment of each specimen in glucose broth, thioglycollate glucose broth, and on blood agar, E.M.B., and SS plates.
3. Salmonella typhosa may be found in typhoid carriers.

Liver Function Tests

General Considerations

The liver is the largest organ in the body. It has a very large factor of safety; 15 per cent of the total liver is capable of maintaining normal liver function. It has many functions, but there is no differentiation of cells according to function. Therefore, if a test of one function reveals liver damage, there is probable interference with all the other functions. However, tests which reveal deficiency of one function do not necessarily imply that all other functions are disturbed in the same degree. Due to the large factor of safety a negative test does not rule out hepatic disease. The tests in this section will be grouped according to functions.

Liver function tests may be divided on the basis of their sensitivity as well as grouped according to function. When the tests are used according to sensitivity, the degree of liver impairment may be determined. Mateer has grouped the liver function tests on the basis of their relative sensitivity as follows:

1. *Most Sensitive Tests.*
   a. Cephalin-cholesterol flocculation test.
   b. Sulfobromophthalein test (5 mg. per kg., 45 min.).
   c. Intravenous hippuric acid test.
   d. Thymol turbidity and flocculation test (slightly less sensitive than first 3 tests).
2. *Tests with Intermediate Sensitivity.*
   a. Oral hippuric acid test (about one half as sensitive as first group).
   b. Urinary urobilinogen test.
3. *Least Sensitive Tests.*
   a. Prothrombin after vitamin K.
   b. Serum albumin.
   c. Oral galactose tolerance test.
   d. Blood cholesterol and esters.

Mateer has also listed the various liver function tests which are best adapted to different types of hepatic problems as follows:

1. *Screening Tests for Early Liver Impairment.*
   a. Cephalin-cholesterol flocculation test.
   b. Sulfobromophthalein test (5 mg. per kg., 45 min.).
   c. Thymol turbidity and flocculation test.
   d. Serum bilirubin.
   e. Intravenous hippuric acid test.
2. *Early Differentiation of Obstructive and Hepatic Jaundice.*
   a. Quantitative urinary urobilinogen test.
   b. Oral galactose tolerance test.
   c. Oral or intravenous hippuric acid test.
   d. Blood prothrombin after several days o parenteral vitamin K therapy.
3. *Acute Hepatitis (Initial Impairment and Progress).*
   a. Serum bilirubin.
   b. Cephalin-cholesterol flocculation test.
   c. Thymol turbidity and flocculation test.
   d. Quantitative urinary urobilinogen test.
   e. Oral or intravenous hippuric acid test.
4. *Chronic Hepatitis and Hepatic Cirrhosis.*
   a. Sulfobromophthalein test (5 mg. per kg., 45 min.).
   b. Cephalin-cholesterol flocculation test.
   c. Thymol turbidity and flocculation test.
   d. Oral hippuric acid test.
   e. Serum albumin.
   f. Blood prothrombin.
5. *Metastatic Carcinoma of Liver.*
   a. Sulfobromophthalein test (5 mg. per kg., 45 min.).
   b. Oral hippuric acid test.
6. *Maximum Liver Impairment (unfavorable prognosis).*
   a. Low serum albumin (below 2.5 gm. per 100 cc.).
   b. Low oral hippuric acid (below 15 per cent of normal).
   c. Low blood prothrombin (below 15 per cent).
   d. Very high sulfobromophthalein retention (60 min. reading).

Tests for External Secretory Function

One of the best known functions of the liver is that associated with the formation and excretion of bile. The important components of bile are bile pigments, bile salts, cholesterol, lecithin, and phosphatase. Tests for the excretory function of the liver are concerned chiefly with bile pigments which are a product of the metabolism hemoglobin. Under normal conditions erythcytes are broken down by the cells of the reulo-endothelial system which releases hematin d globin. Hematin (porphyrin and iron) is rther hydrolized to a certain type of bilirubin signated as hemobilirubin which in the blood combined with serum proteins. Hemobilirubin es not pass through the kidney filter and does t appear in the urine. In the liver it passes om the blood in the sinusoids to the Kupffer's lls and thence to the parenchymatous cells here it is changed to a different type of bilibin known as cholebilirubin, which is excreted the bile and when present in the blood is exeted by the kidneys. This cholebilirubin is nverted to urobilinogen by bacterial action in e intestinal tract. The greater part of the urolinogen is excreted in the feces, but a portion it is reabsorbed into the blood stream. Most the reabsorbed urobilinogen is re-excreted by e liver as bilirubin; smaller amounts are either nverted to hemoglobin or excreted by the dneys as urobilinogen or as urobilin.

. Icterus Index (Meulengracht's Method).

Principle: The yellow color of blood serum is due chiefly to the presence of hemobilirubin or cholebilirubin. The intensity of yellow color of the serum is compared with a standard potassium dichromate solution. The color intensity is not directly proportional to the amount of bilirubin present. Cholebilirubin in serum gives a deeper color than a corresponding amount of hemobilirubin. Therefore, the icterus index does not always agree with the Van den Bergh test.

. *General Considerations.*

Test must be made on fasting blood to avoid milky serum.

The blood must be free from hemolysis.

Patient should not eat any food containing yellow pigment (especially carrots) for 24 to 48 hours before the test.

. *Method Using Permanent Standards.*

Obtain 5 to 6 cc. of venous blood and allow to clot.

Centrifuge and then place the serum in a test tube of the same bore as the standards.

Compare with standards.

If the serum contains over 20 units, it should be diluted with 0.85% sodium chloride or 5% sodium citrate solution so it will come within the range below 20 units.

. *Colorimetric Method.*

The serum should be diluted with 0.85% sodium chloride or 5% sodium citrate solution to approximate the color of the standard.

2. Compare in a colorimeter with standard set at 15.
3. *Calculation:*

$$\frac{RS}{RU} \times \text{dilution} = \text{icterus units.}$$

E. *Photoelectric Colorimeter Method.*

1. Place 9 cc. of 0.1 N HCl in a colorimeter tube, add 1 cc. of clear serum, and mix by twirling.
2. Pipette 10 cc. of 0.1 N HCl into another tube for the blank.
3. Adjust the galvanometer to 100 using the blank and filter No. 420, and then read the unknown.
4. Obtain the icterus units from the table of values.
5. If over 20 units, dilute further with 0.1 N HCl and read. Correct the value for dilution used.
6. *Calibration of Standard Curve:*
   a. Make a dilute standard solution (0.1%) by placing 10 cc. of the 1% stock solution of potassium dichromate in a 100 cc. volumetric flask, add 2 drops of conc. sulfuric acid, and dilute to volume with distilled water.
   b. Pipette the dilute standard solution and distilled water containing 2 drops of conc. sulfuric acid per 100 cc. into 9 colorimeter tubes in the following proportions:

cc. of dilute standard solution	cc. of acidified distilled H_2O	Icterus units
0.1	9.9	1
0.3	9.7	3
0.5	9.5	5
0.8	9.2	8
1.0	9.0	10
1.5	8.5	15
2.0	8.0	20
3.0	7.0	30
0.0	10.0	Blank

   c. Adjust the galvanometer to 100 with the blank using filter No. 420.
   d. Mix the contents of each tube and read.
   e. Repeat using the same dilute standard solution; also repeat using new dilute standard solutions made from a new stock standard solution.
   f. Plot the average of the galvanometer readings for each tube on semilogarithmic graph paper.
   g. List in a table of values the icterus units for each division on the galvanometer.

F. *Solutions.*

1. *Stock Standard Solution*—1%.
   a. Place 1 gm. of c.p. potassium dichromate in a 100 cc. volumetric flask.
   b. Dissolve in about 70 cc. of distilled water,

add 2 drops of conc. sulfuric acid, and dilute to volume.

c. Place in a glass-stoppered brown bottle.

2. *Permanent Standards.*

a. Pipette the stock standard solution and distilled water containing 2 drops of conc. sulfuric acid per 100 cc. into 20 test tubes in the following proportions:

Tube	cc. of stock standard solution	cc. of acidified distilled H_2O	Icterus units*
1	5.0	5.0	50
2	4.5	5.5	45
3	4.0	6.0	40
4	3.5	6.5	35
5	3.0	7.0	30
6	2.5	7.5	25
7	2.0	8.0	20
8	1.6	8.4	16
9	1.4	8.6	14
10	1.2	8.8	12
11	1.0	9.0	10
12	0.9	9.1	9
13	0.8	9.2	8
14	0.7	9.3	7
15	0.6	9.4	6
16	0.5	9.5	5
17	0.4	9.6	4
18	0.3	9.7	3
19	0.2	9.8	2
20	0.1	9.9	1

* One unit is equivalent to 0.05 mg. of bilirubin.

b. Fill 20 small test tubes of uniform bore (8 mm.) two-thirds full from the above solutions.

c. Close by sealing the upper end of the tubes in a flame or by inserting cork stoppers and sealing with paraffin.

d. Keep in a dark place.

e. New standards should be made every 6 months.

3. *Standard Solution for Colorimetric Method.*

a. Place 10 cc. of the stock standard solution in a liter volumetric flask, add about 700 cc. of distilled water, add 2 drops of conc. sulfuric acid, and dilute to mark.

b. This is a 0.01% solution of potassium dichromate and represents 1 unit.

c. Solution will keep 6 months in the dark.

4. *Sodium Citrate Solution—5%.*

a. Dissolve 50 gm. of sodium citrate, c.p., in water and dilute to 1 liter.

b. If turbid, allow to stand several days and filter.

c. Add a few cc. of chloroform as a preservative.

G. *Interpretation of Serum Icterus Index Findings.*

1. *Normal Values:* 4-6 units.
2. *Increased in:*

The icterus index may increase to 15 un without any clinical signs of jaundice.

Latent jaundice (6-15 units)
Infectious hepatitis
Toxic jaundice
Obstructive jaundice
Hemolytic jaundice
Pernicious anemia
Acute yellow atrophy

3. *Decreased in:*

Hypochromic microcytic anemia (2-4 units

II. Van den Bergh (Thannhauser and Anderson's Colorimeter Method).

A. ***Principle:*** The test depends on the couplin of diazobenzolsulfochloride (Ehrlich's diaz reagent) with the bilirubin of the serum an the production of a red color due to the formation of acetophenolazobilirubin. The tim of the appearance and depth of this colo indicates the type and degree of the bilirubinemia. If the bilirubin has passed throug the liver (cholebilirubin), it will react directl with the diazo reagent. This is called th *direct* reaction. If the bilirubin has not passe through the liver but is the result of hemolysi (hemobilirubin), it will react only with th diazo reagent after the proteins with whic it is combined have been precipitated wit alcohol. This has been called the indirect reaction but should be designated *total* bilirubi as it includes both types of bilirubin, that giving the direct and the indirect reactions.

B. *General Considerations.*

1. The blood must be free from hemolysis.
2. Dilute serum according to the icterus inde value, a dilution with a reading of about 2 units is preferable.

C. *Method.*

1. Place 2 cc. of clear serum in a 15 cc. centrifuge tube and add 1 cc. of freshly prepare diazo reagent.
2. *Direct Reaction* (Qualitative).
   a. *Prompt*—color change begins within 3 seconds and reaches its full developmen within 1 minute.
   b. *Delayed*—color change begins after 3 seconds and requires 10 minutes to reac its maximum.
   c. *Biphasic*—color appears promptly but requires 10 minutes to reach its maximum
   d. If no color develops, there will not b enough bilirubin present to make it wort while doing an indirect reaction.
3. *Indirect Reaction* (Total Bilirubin).

a. Add 2 cc. of saturated ammonium sulfate solution and 10 cc. of 95% alcohol to the centrifuge tube used for the direct reaction.

b. Stopper, mix by inverting, then centrifuge.

c. *Standard.*
   1) Pipette 5 cc. of dilute standard solution into a 60 cc. separatory funnel; add 5 cc. of 20% potassium thiocyanate solution and 20 cc. of ether.
   2) Shake well and then drain off the lower layer of fluid leaving the supernatant ether.
   3) Standardize the colorimeter immediately with the ether extract.

d. Read the unknown (top alcoholic extract) immediately with the standard set at 20 mm.

e. *Calculation:*

$$\frac{RS}{RU} \times 0.5 \times \frac{15}{2} = \frac{75}{RU} = \text{mg.\% of bilirubin.}$$

Solutions.

Standard Artificial Bilirubin.

a. *Stock solution.*
   1) Dissolve 0.1508 gm. of ferric ammonium alum in 50 cc. of conc. hydrochloric acid in a 100 cc. volumetric flask and make up to volume with distilled water.
   2) This solution keeps indefinitely in a glass-stoppered brown bottle.

b. *Dilute standard solution.*
   1) Add 25 cc. of conc. hydrochloric acid to 10 cc. of the stock solution in a 250 cc. volumetric flask and dilute to volume with distilled water.
   2) This solution keeps about 6 months.
   3) It represents 0.5 mg. of bilirubin in 100 cc. of serum.

Diazo Reagent.

a. *Solution A*—dissolve 5 gm. of sulfanilic acid in 50 cc. of conc. hydrochloric acid in a liter volumetric flask and dilute to volume with distilled water.

b. *Solution B*—dissolve 0.5 gm. of sodium nitrite in 100 cc. of distilled water and keep in a brown bottle in the refrigerator.

c. Prepare the diazo reagent fresh immediately before performing the test by adding 24.2 cc. of Solution A to 0.8 cc. of Solution B.

Saturated Solution of Ammonium Sulfate—100 gm. to 100 cc. of distilled water.

Potassium Thiocyanate Solution—20%.

Interpretation of Serum Van den Bergh Findings (See Table 18, p. 110).

1. *Normal Total Bilirubin:* 0.2-0.8 mg. per cent Bilirubin found normally in serum is produced by the destruction of hemoglobin in the reticulo-endothelial system. This type of bilirubin gives only the indirect reaction.

2. *Hyperbilirubinemia*—there are three general types of disturbances which may act singly or in combination to produce hyperbilirubinemia.
   a. Conditions in which bilirubin is produced in excess of capacity of the liver to excrete it (hemolytic type of jaundice).
      1) Found in congenital hemolytic jaundice, icterus neonatorum, erythroblastosis fetalis, Cooley's anemia, acute hemolytic anemia, polycythemia, pernicious anemia, sickle cell anemia, malaria, paroxysmal hemoglobinuria, hemolytic septicemia, incompatible blood transfusion, toxic conditions from phenylhydrazine and sulfonamide drugs.
      2) This type alone gives only the indirect reaction.
   b. Conditions in which the rate or production of bilirubin is not increased, but because of toxic or infectious injury to the liver cells and finer bile passages it accumulates in the blood stream (hepatogenous jaundice).
      1) Infectious type of jaundice is found in infectious hepatitis, yellow fever, and spirochetal jaundice.
      2) Toxic type of jaundice is caused by arsphenamine, mercury, chloroform, and carbon tetrachloride poisoning, exposure to Roentgen ray, eclamptic hepatitis, and acute yellow atrophy of the liver.
      3) The early phase of this type gives only the indirect reaction, but as the damage to the liver progresses the direct reaction appears.
   c. Conditions in which obstruction to the larger bile passages causes a reflux of bilirubin into the blood (obstructive jaundice).
      1) Obstruction of intrahepatic bile ducts—cholelithiasis or impaction of parasites.
      2) Occlusion of the hepatic ducts due to inflammation, tumor, or congenital atresia or stenosis.
      3) Occlusion of the common bile duct by tumor or concretion.
      4) This type when uncomplicated gives only the direct reaction.

3. *Hypobilirubinemia* is found in hypochromic microcytic anemia.

TABLE 18. DIFFERENTIAL DIAGNOSIS OF JAUNDICE

	Normal	Hemolytic Jaundice	Obstructive Jaundice	Hepatogenou Jaundice
Van den Bergh reaction	Indirect	Indirect	Direct	Indirect
Total serum bilirubin	0.2 - 0.8 mg. %	Increased	Increased	Increased
Fecal urobilinogen		Increased	Decreased or none	Decreased or normal
Adult	30 - 200 mg. per 100 gm.			
Children 3 - 11 yrs.	2 mg. per 100 gm.			
Infants to 2 yrs.	2 - 5 mg. per 100 gm.			
Urinary urobilinogen	0.2 - 3 mg. per 24 hr. or less than 1.0 E.U. in 2 hr.	Increased	Decreased or none	Increased or normal
Urinary bilirubin	None	None	Present	Present

4. *Renal threshold* for cholebilirubin is 1.75 to 2 mg. per cent in the serum.

III. Van den Bergh (Evelyn and Malloy's Photoelectric Colorimeter Method).

A. ***Principle:*** The diazotized sulfanilic acid is coupled with bilirubin in either an aqueous or methyl alcohol medium to produce a pink compound known as azorubin or azobilirubin; the color being measured in the photoelectric colorimeter using a green filter (540).

B. ***General Considerations.***

1. Either serum or plasma may be used.
2. Hemolysis should be avoided if possible, but fairly accurate results can be obtained even in the presence of a moderate degree of hemolysis.
3. Glassware should be scrupulously clean to avoid bubbles.

C. ***Method.***

1. All solutions must be added to the tubes in the exact order as given.
2. Label 4 colorimeter tubes 1 to 4 and add the following:

   Tube 1. Direct Blank.
   5 cc. of distilled water
   1 cc. of diazo blank solution
   Tube 2. Direct Reaction.
   5 cc. of distilled water
   1 cc. of diazo reagent
   Tube 3. Indirect Blank.
   5 cc. of absolute methyl alcohol
   1 cc. of diazo blank solution
   Tube 4. Indirect Reaction.
   5 cc. of absolute methyl alcohol
   1 cc. of diazo reagent

3. Make a 1-10 dilution of serum by diluting 2 cc. with 18 cc. of distilled water.
4. Add 4 cc. of the diluted serum to each of the colorimeter tubes in the following order: 1, 3, 4, and 2.
5. Mix the contents gently but thoroughly by twirling the tubes.
6. The tubes should be clear and free from bubbles before being inserted into the colorimeter. If bubbles are present, they must
   dislodged by gently tapping the tube.
7. Using tube 1 as the blank for the dire
   reaction, set the galvanometer at 100 wi
   filter No. 540.
8. Read tube 2 at the end of 1 minute (tim
   from beginning of addition of serum) a
   again at 30 minutes.
9. If the galvanometer reading is below 10, a
   10 cc. of distilled water to tubes 1 and
   repeat readings, and multiply final val
   by 2.
10. Using tube 3 as the blank for the indire
    reaction, set the galvanometer at 100 a
    read tube 4 at the end of 30 minutes. If t
    galvanometer reading is below 10, add 10 c
    of 50% methyl alcohol to each tube (3 a
    4), repeat readings, and multiply final val
    by 2.
11. *Calculation:*

$$\frac{100 \times \text{L value of tube 2}}{\text{K}} = \text{mg.\% of direct bilirub}$$

$$\frac{100 \times \text{L value of tube 4}}{\text{K}} = \text{mg.\% of total (indirect) bilirubin}$$

L value = 2 — log of the galvanometer reading.
K = 6.72 for the Evelyn instrument.

12. A calibration curve can be determined f
    other instruments in the following manne
    a. Make a stock standard solution by placi
       10 mg. of pure bilirubin, accurate
       weighed, in a 100 cc. volumetric fla
       and adding chloroform to volume.
    b. Make a dilute standard solution by pla
       ing 10 cc. of the stock solution in a 1
       cc. volumetric flask and diluting to vo
       ume with methyl alcohol (1 cc. = 0.0
       mg. of bilirubin).
    c. Pipette 1 cc. of the diazo reagent int
       each of 6 colorimeter tubes.
    d. Add the amount of dilute standard sol
       tion and methyl alcohol according to th
       following:

Tube	cc. of diazo reagent	cc. of dilute standard solution	cc. of methyl alcohol	mg. of bilirubin in 100 cc. of serum
1	1	1	8	2.5
2	1	2	7	5.0
3	1	4	5	10.0
4	1	6	3	15.0
5	1	8	1	20.0
6	1	0	9	Blank

e. Allow 30 minutes for the colors to develop; adjust the galvanometer to 100 with the blank and obtain the readings of the standards.

f. Repeat test at least twice; also repeat with at least 2 new stock solutions in the same manner.

g. Plot the average of the galvanometer readings for each tube on semilogarithmic graph paper.

h. List in a table the values (mg. per 100 cc. of blood) for each division on the galvanometer.

Solutions.

Diazo Reagent.

a. *Solution A*—dissolve 1 gm. of sulfanilic acid in a liter of distilled water containing 15 cc. of conc. hydrochloric acid.

b. *Solution B*—dissolve 0.5 gm. of sodium nitrite in 100 cc. of distilled water and keep in a brown bottle in the refrigerator. Discard if solution turns yellow.

c. Prepare the diazo reagent immediately before performing the test by adding 0.3 cc. of Solution B to 10 cc. of Solution A.

Diazo Blank Solution.

a. Place 15 cc. of conc. hydrochloric acid in a liter volumetric flask.

b. Make up to volume with water.

Interpretation of Serum Van den Bergh Findings (See Table 18).

Normal Total Bilirubin: 0.2–0.8 mg. per cent.

The 3 types of reactions can be determined by finding the ratio of the 1 and 30 minute direct reaction (D) to the 30 minute total bilirubin (T).

a. *Prompt Reaction.*

$$\frac{\text{1 min. D.}}{\text{30 min. T.}} = 40 \text{ to } 70\%.$$

$$\frac{\text{30 min. D}}{\text{30 min. T.}} = 60 \text{ to } 100\%.$$

b. *Delayed Reaction.*

$$\frac{\text{1 min. D.}}{\text{30 min. T.}} = 0 \text{ to } 25\%.$$

$$\frac{\text{30 min. D.}}{\text{30 min. T.}} = 5 \text{ to } 55\%.$$

c. *Biphasic Reaction.*

$$\frac{\text{1 min. D.}}{\text{30 min. T.}} = 25 \text{ to } 40\%.$$

$$\frac{\text{30 min. D.}}{\text{30 min. T.}} = 25 \text{ to } 60\%.$$

3. In normal blood the ratio of direct to total bilirubin has no significance.
4. In obstructive jaundice the 30 minute direct is 60 to 100 per cent of the total (prompt reaction).
5. In pure hemolytic jaundice without liver cell damage, the 30 minute direct is less than 25 per cent of the total (delayed reaction).
6. In hepatogenous jaundice the 30 minute direct is 25 to 60 per cent of the total (biphasic reaction).
7. For significance of hyper- and hypo-bilirubinemia, see interpretation of Thannhauser and Anderson's Van den Bergh test, page 109.

IV. Bilirubin Excretion Test (Von Bergman's Method).

A. ***Principle:*** The excretion of ingested bilirubin is solely a function of the liver. By increasing the amount of bilirubin in the blood stream by the intravenous injection of pure bilirubin, the hepatic cells are taxed by this increased load. By studying the rate of removal of the added bilirubin, evidence of hepatic damage is demonstrated.

B. ***General Considerations.***

1. The test is useless and contraindicated in any patient with hyperbilirubinemia.
2. It is the most sensitive indicator of slight impairment of liver function.
3. The sodium carbonate solution should not be boiled more than a few seconds to prevent its producing a reaction when injected.

C. ***Method.***

1. Weigh out an amount of bilirubin (Eastman Kodak Co.) equivalent to 1 mg. per kilogram body weight. (Never use more than 70 mg.)
2. Dissolve the bilirubin in 15 cc. of 0.1 molar solution of sodium carbonate which has been brought to the boiling point and cooled to 80°C. It forms a clear iodine-colored solution.
3. By venipuncture, withdraw 10 cc. of blood (control) and place in a test tube.
4. Through the same needle inject the bilirubin solution (cooled to body temperature) slowly, avoiding leakage.

5. After 5 minutes and again after 4 hours, withdraw 10 cc. of blood from a vein in opposite arm and place in test tubes. Avoid hemolysis.
6. Centrifuge the 3 blood samples.
7. Determine the total bilirubin in each of the 3 serums by Evelyn and Malloy's photoelectric colorimeter method for the Van den Bergh test.
8. *Calculation:*
    a. The difference between the 4 hour sample and the control is the amount of bilirubin retained (A).
    b. The difference between the 5 minute sample and the control is considered 100 per cent of the injected bilirubin (B).
    c. Then (A) divided by (B) gives the per cent of bilirubin retained.

D. *Solutions.*

1. *Sodium Carbonate* (0.1 M Na_2CO_3)—dissolve 1.06 gm. in 100 cc. of sterile triple distilled water.
2. See solutions for Van den Bergh test.

E. *Interpretation of the Bilirubin Excretion Test.*

1. *Normal Values:* Less than 6 per cent retention in 4 hours.
2. *Increased in:* Cirrhosis, malignant tumors of the liver, and diffuse hepatitis.

V. Sulfobromophthalein or Bromsulfalein Test (Green's Modified Method).

A. ***Principle:*** Sulfobromophthalein sodium (bromsulfalein) injected into the blood stream is removed by reticulo-endothelial cells of the liver and excreted by parenchymal cells into the bile within a short time after injection. The degree of dye retention is measured by a colorimetric method.

B. *General Considerations.*

1. Patient should be instructed to come to the laboratory in the morning without having taken any food since the evening before.
2. Injection of the dye produces a transient reaction in a few patients consisting of headache, faintness, and chills. However, there are no serious after effects.
3. If the blood, withdrawn for the test, is badly hemolyzed and the comparator block method is used, extract the serum with an equal volume of acetone and centrifuge. Some dye is lost, but alkalinization of the clear supernatant fluid permits approximation of the amount of dye. Hemolysis does not interfere in the photoelectric colorimeter method.
4. Not applicable or accurate in the presence of jaundice when the comparator block method is used. The sulfobromophthalein test should not be used in cases of obstructive jaundic for two reasons: (a) the retention of dye due to obstruction and is not a reliable te for impaired liver function, and (b) ther may be danger to the patient in retention o a large amount of the dye.
5. Ampules containing 3 cc. of a 5% sterile solution of bromsulfalein may be obtained fro Hynson, Westcott, and Dunning (Baltimore, Md.).
6. The bromsulfalein may be given in a dose of either 5 mg. or 2 mg. per kilogram of body weight; however, the 5 mg. dose is considered the more sensitive.

C. *Administration of Dye.*

1. Weigh the patient and calculate the dosage on the basis of 5 mg. of bromsulfalein per kilogram of body weight. Each 10 kilograms requires 1 cc. of sterile 5% dye solution.
2. Inject the correct amount of the dye into an arm vein. The injection should be sufficiently slow to occupy 1 minute and the dye should not be allowed to escape outside the vein.
3. Forty-five minutes after the injection, withdraw 10 cc. of blood in a dry syringe from a vein of the opposite arm and place the blood in a test tube to clot. Blood is sometimes drawn 60 minutes after the injection of dye and the amount of dye retention is of prognostic value in severe liver damage.
4. If 2 mg. of dye per kg. is given, obtain blood at the end of 20 minutes.

D. *Estimation of Dye Retention.*

1. *Comparator Block Method.*
    a. After centrifugation of the blood, pipette the clear serum into 2 small test tubes having the same diameter as the standard tubes.
    b. To one tube add 1 drop of 10% NaOH to bring out the color of the dye and to the other 1 drop of 5% HCl to clarify the serum or discharge a faint redness if present.
    c. After 10 minutes place the tubes in a comparator box and compare with a series of standards.
    d. The tube of clear acidified serum is placed in front of the standard and this is compared with the alkalinized serum which has a tube of distilled water placed behind it.
    e. Compare with different standard tubes until the nearest match is obtained.
    f. The percentage of dye is marked on the standard tube. When the 5 mg. dose is used, the percentage is divided by 2.5 because the standards are made on the basis

of giving 2 mg. per kilogram of body weight as was given in the original method.

g. Standards may be obtained from Hynson, Westcott, and Dunning.

Photoelectric Colorimeter Method (Gaebler).

a. After centrifugation of the blood, place 0.5 cc. of the clear serum in each of 2 colorimeter tubes.

b. Add 2.5 cc. of distilled water to each tube and mix.

c. Add 3 cc. of 0.1 N NaOH to 1 tube and mix.

d. Add 3 cc. of 0.1 N HCl to the other tube and mix; this tube is used as the blank to set the galvanometer at 100 using filter No. 565.

e. Obtain the galvanometer reading for the tube containing the 0.1 N NaOH and calculate the amount of dye retention.

f. *Calculation:*

1) The mg. per cent of dye present in the serum may be obtained from the table of values or may be calculated from the following formula:

$$\frac{L - 0.005}{0.111} = \text{mg.\% of dye.}$$

$$L = 2 - \log \text{ of the galvanometer reading.}$$

2) If the 5 mg. dose is used, the mg. per cent of dye is multiplied by 10 to obtain the per cent retention, since 10 mg. per cent is considered 100 per cent retention.

3) If the 2 mg. dose is used, the mg. per cent of dye is multiplied by 25 to obtain the per cent retention, since 4 mg. per cent is considered 100 per cent retention.

4) Values should be reported to the nearest full per cent of retention.

g. *Calibration of standard curve.*

1) The bromsulfalein-free serum used to make the standard curve should be a mixture of a large number of serums (30 to 40) and tested for an abnormal amount of pigment as follows:

a) Prepare a serum tube:
0.5 cc. of serum.
2.5 cc. of distilled water.
3.0 cc. of 0.1 N NaOH.

b) Prepare a blank in exactly the same manner except use 0.1 N HCL instead of 0.1 N NaOH.

c) Set the galvanometer at 100 using the blank and filter No. 565, then read the serum tube which should read between 98 and 100.

d) If the serum tube reads lower than 98, there is too much pigment present and a different pooled serum must be tested.

2) Stock dye solution (50 mg.%)—place 1 cc. of the 5% bromsulfalein dye in a 100 cc. volumetric flask and dilute to volume with distilled water.

3) Prepare dilute dye solutions as follows:

cc. of stock dye solution	Dilute to volume with water	mg.% of bromsulfalein
0.5	100 cc.	0.25
1.0	100 cc.	0.5
1.0	50 cc.	1.0
2.0	50 cc.	2.0
4.0	50 cc.	4.0

4) Add the following to a colorimeter tube for each of the dilute dye solutions.
0.5 cc. of bromsulfalein-free serum.
0.5 cc. of a dilute dye solution.
2.0 cc. of distilled water.
3.0 cc. of 0.1 N NaOH.

5) Prepare a blank as follows:
0.5 cc. of bromsulfalein-free serum.
2.5 cc. of distilled water.
3.0 cc. of 0.1 N HCl.

6) Set the galvanometer at 100 using the blank and filter No. 565 and then read the tubes containing the dilute dye solutions.

7) Repeat several times using the same dilute dye solutions and then repeat with several new stock dye solutions.

8) Average the galvanometer readings for each concentration of dye and plot on semilogarithmic graph paper.

9) Make a table of values according to the mg. per cent of dye for each galvanometer division.

E. *Interpretation of the Sulfobromophthalein Test.*

1. *Normal Values:*

a. *For the comparator block method.*

1) No dye present at 45 minutes with the 5 mg. dose.

2) No dye present at 20 minutes with the 2 mg. dose.

b. *For the photoelectric colorimeter method.*

1) Retention of 4 per cent or less of dye at 45 minutes with the 5 mg. dose or a retention of 2 per cent at 60 minutes.

2) Retention of 2 per cent or less of dye at 20 minutes with the 2 mg. dose.

2. The retention of dye indicates liver damage in proportion to the degree of retention.

3. The test is most valuable in liver disease without jaundice.

4. When jaundice is present, the concentration

of dye is a measure of biliary retention rather than of hepatic insufficiency.

5. Both obstructive and hepatogenous jaundice show marked retention.
6. Vascular stasis (passive congestion of the liver) will cause retention of dye.

VI. Fecal Urobilinogen (Watson's Method).

A. ***Principle:*** Preformed urobilin is changed to urobilinogen by ferrous sulfate and sodium hydroxide (ferrous hydroxide). The urobilinogen combines with Ehrlich's reagent (acid solution of para-dimethylaminobenzaldehyde) to produce a red color. This is the so-called aldehyde reaction and depends on the presence of the pyrrole group. The color is compared with an artificial standard either in a visual colorimeter or in a photoelectric colorimeter.

B. ***General Considerations.***

1. Specimen should be taken to the laboratory at once and kept in the refrigerator until tested.
2. Results of determinations on single specimens are reported in milligrams of urobilinogen per 100 grams of feces.
3. Results of specimens collected during 24 or more hours are reported in milligrams per 24 hour excretion.

C. ***Colorimetric Method.***

1. Thoroughly mix the feces either in the carton or in a mortar.
2. Weigh out 10 gm. of the feces in a small evaporating dish.
3. Grind thoroughly into a paste with a 10 to 20 cc. portion of a 90 cc. amount of distilled water and transfer to a mortar.
4. Wash the evaporating dish with portions of the water and transfer to the mortar.
5. Mix thoroughly by grinding.
6. Allow the mixture to stand for a short time, then decant the supernatant fine suspension of feces into a liter Erlenmeyer flask containing 100 cc. of freshly prepared ferrous sulfate solution (20 gm. of ground $FeSO_4 \cdot 7H_2O$ crystals and 92 cc. distilled water).
7. Grind the remaining material in the mortar repeatedly with small portions from 200 cc. of distilled water; pour all of these into the large Erlenmeyer flask until the mortar is finally washed clean.
8. Add 100 cc. of 10% NaOH slowly while gently shaking the flask.
9. After thorough mixing, cork the flask and set aside in the dark for 1 hour.
   a. If the supernatant solution is not almost clear at the end of the hour, the r duction should be continued for anoth hour.
   b. If then the reduction is not comple a measured portion of the supernata fluid should be mixed with fresh ferro hydroxide as described above and l stand another hour.
10. If the feces appeared acholic (clay-colored modify the above procedure by using onl 25 cc. of 20% ferrous sulfate solution, 10 cc. of distilled water to grind the sample, an 25 cc. of 10% NaOH solution.
11. After complete reduction filter the mixtur in the Erlenmeyer flask through filter paper (The remainder of the test must not be carried out in a brightly lighted room, as ligh will destroy some of the urobilinogen.)
12. *Preliminary Test.*
   a. Place 2 to 3 cc. of the filtrate in a tes tube and acidify with an equal amoun of modified Ehrlich's reagent.
   b. Add 4 to 6 cc. of a saturated aqueou solution of sodium acetate and notice th intensity of the developing color.
   c. If the color is very intense 1 cc. of filtrat should be used in the quantitative determination, if moderately intense use 2 cc., if pale red use 5 to 10 cc., if fain 15 to 25 cc., if absent 50 cc.
13. Dilute the amount of filtrate decided upon t 25 cc. (if less than that amount) and plac in a small separatory funnel.
14. Cover with approximately 50 cc. of pur petroleum ether which has been acidifie with 5 cc. of glacial acetic acid.
15. Immediately shake the mixture vigorousl for several seconds.
16. Allow the petroleum ether to separate; if a emulsion forms, it can be broken by the addition of further acetic acid or 1 to 2 cc. o 95% alcohol.
17. Collect the aqueous fraction in another separatory funnel; then decant the petroleu ether into a clean separatory funnel.
18. The aqueous fraction is extracted twice mor with 25 cc. portions of petroleum ethe which is decanted as described above.
19. Wash the combined petroleum ether extractions once with a small amount of distilled water. The water is discarded.
20. Extract the urobilinogen from the petroleu ether by vigorously shaking for 1 minut with 2 cc. of Ehrlich's reagent.
21. Add 6 cc. of saturated aqueous solution o sodium acetate which brings out the maxi mum color in the aqueous solution.
22. Shake vigorously.

Color due to indole or skatol disappears as the hydrochloric acid forms acetic acid from the sodium acetate. Any excess of aldehyde returns to the petroleum ether.

Separate off the colored solution into a 100 cc. graduated cylinder.

Again shake the petroleum ether with the same amount of Ehrlich's reagent and sodium acetate solution.

Add the colored solution to the above.

If more than a very faint color develops, this procedure must be repeated until it is certain that the extraction of urobilinogen is complete.

Add water to the colored solution to make a volume convenient in calculation and also to obtain a color comparable to the standard. Mix well and compare immediately in a colorimeter with the standard set at 20 mm.

Calculation:

$$\frac{RS}{RU} \times 0.5 \times \frac{\text{cc. ferrous hydroxide mixture}}{\text{grams of feces}} \times \frac{\text{cc. final solution}}{\text{cc. filtrate used}} = \text{mg. per 100 gm.}$$

Photoelectric Colorimeter Method.

The test is performed as described for the colorimetric method.

Place 10 cc. of the well-mixed final solution in a colorimeter tube.

Prepare a blank in another colorimeter tube consisting of 9 cc. of the sodium acetate solution and 3 cc. of Ehrlich's reagent.

Set the galvanometer at 100 using the blank and filter No. 565.

Read the unknown and obtain the mg. per cent of urobilinogen from the table of values.

Calculation:

$$\text{mg.\%} \times \frac{500}{10} \times \frac{\text{Vol. final sol.}}{\text{Vol. filtrate used}} = \text{mg. per 100 gm.}$$

Calibration of Standard Curve.

a. Make a stock standard dye solution as follows:

Pontacyl Carmine 2B	5 mg.
Pontacyl Violet 6R (150 per cent)	95 mg.

Make up to 1 liter with 0.5% acetic acid.
(Obtain the dyes from E. I. du Pont de Nemours Company, Wilmington, Del.)

b. Make a dilute standard dye solution by placing 20.4 cc. of the stock solution in a 100 cc. volumetric flask and diluting to volume with 0.5% acetic acid.

c. This dilute solution is equivalent to 0.6 mg. of urobilinogen in 100 cc.

d. Pipette the following amounts of dilute dye solution and 0.5% acetic acid in a series of colorimeter tubes.

cc. of dilute dye solution	cc. of 0.5% acetic acid	Equivalent to mg. of dye per 100 cc.	Equivalent to mg. per cent of urobilinogen
0.84	19.16	0.085	0.025
1.67	18.33	0.17	0.05
3.34	16.66	0.34	0.10
5.00	15.00	0.51	0.15
6.67	13.33	0.68	0.20
8.34	11.66	0.85	0.25
10.00	10.00	1.02	0.30
13.34	6.66	1.36	0.40
16.66	3.34	1.70	0.50
20.00	0.00	2.04	0.60
0.00	20.00	Blank	0.00

e. Set the galvanometer at 100 with the blank using filter No. 565; then read the tubes containing the dye solution after they are thoroughly mixed by shaking.

f. Repeat a sufficient number of times so that the average of the readings for each dilution will give a straight line when plotted on semilogarithmic graph paper.

g. Make a table of values for the mg. per cent of urobilinogen for each galvanometer division.

E. *Solutions.*

1. *Modified Ehrlich's Reagent.*
   a. Place 0.7 gm. pure para-dimethylaminobenzaldehyde (Pfanstiehl) in a 500 cc. Erlenmeyer flask.
   b. Add 150 cc. conc. HCl and 100 cc. distilled water.
2. *Purified Petroleum Ether.*
   a. Let petroleum ether stand for several days over conc. sulfuric acid in a large Florence flask. Keep stoppered and shake occasionally.
   b. Wash again with conc. sulfuric acid and repeatedly with distilled water.
   c. Wash once with 10% NaOH and then 3 times with distilled water.
   d. Filter through Whatman No. 50 filter paper which has been moistened with petroleum ether.
   e. Distill the ether by suspending the flask in boiling water on an electric plate (no flame).
   f. This ether may be used over and over again in the test without repeating the purification.
3. *Saturated Sodium Acetate Solution.*
   a. The sodium acetate ($NaC_2H_3O_2$) must be chemically pure.
   b. Dissolve about 150 gm. in 100 cc. of distilled water.
4. *Standard* (Terwen).
   a. Place 1 cc. of 0.05% alcoholic solution of phenolphthalein in a 100 cc. volumetric flask.

b. Add 5 cc. of a saturated solution of sodium carbonate.
c. Make up to volume with distilled water.
d. This standard solution is equivalent to 0.5 mg. of urobilinogen.

F. ***Interpretation of Fecal Urobilinogen Findings.***

1. *Normal Values:* 30-200 mg. per 100 gm. of feces.
2. *Increased in hemolytic jaundice* due to increased hemolysis of erythrocytes producing more bilirubin which results in an increased amount of urobilinogen formed in the intestine.
3. *Decreased in obstructive jaundice* when either no bile or a decreased amount of bile reaches the intestine to be changed to urobilinogen. In complete obstruction practically none is found in the feces, while in partial obstruction a small amount may be present.
4. *Normal or decreased in hepatogenous jaundice* depending on whether liver cells are capable of secreting a normal amount of bile.

VII. Urine Urobilinogen (Watson's Method).

A. ***Principle:*** See Watson's method for fecal urobilinogen.

B. ***General Considerations.***

1. Single specimens should be tested immediately after being voided.
2. Twenty-four hour specimens are collected in a brown bottle to which has been added about 100 cc. of purified benzin (petroleum ether) and 5 gm. of anhydrous sodium carbonate. Keep in the refrigerator during the collection of the specimens.
3. Occasionally large quantities of protein in urine cause turbidity after the addition of Ehrlich's reagent. It can be removed by mixing equal quantities of urine and a 3% solution of sulfosalicylic acid and filtering.

C. ***Colorimetric Method.***

1. Shake urine to mix and pour into a liter graduate.
2. Measure volume of urine after separation of the petroleum ether which had been added as a preservative.
3. Place 50 cc. of urine in a 125 cc. Erlenmeyer flask and add 25 cc. of a freshly prepared 20% ferrous sulfate solution (5 gm. of $FeSO_4 \cdot 7H_2O$ dissolved in 23 cc. of distilled water).
4. Add 25 cc. of 10% NaOH with thorough mixing.
5. Let stand for 1 hour in the dark and then filter. (The remainder of the test must not be carried out in a brightly lighted room, as light will destroy some of the urobilinogen.)
6. Beginning with the preliminary test, continue as described under Watson's colorimetric method for fecal urobilinogen.
7. *Calculation:*

$$\frac{\text{RS}}{\text{RU}} \times 0.5 \times \frac{\text{cc. ferrous hydroxide mixture} + \text{urine}}{\text{vol. of urine used}}$$
$$\times \frac{\text{cc. final solution}}{\text{cc. filtrate used}} \times \frac{\text{total vol. of urine}}{100}$$
$$= \text{mg. in 24 hours.}$$

D. ***Photoelectric Colorimeter Method.***

1. Follow directions for the colorimetric method of urine urobilinogen and then follow directions for the photoelectric colorimeter method for fecal urobilinogen.
2. *Calculation:*

$$\text{mg.\%} \times \frac{100}{50} \times \frac{\text{vol. of final sol.}}{\text{vol. of filtrate used}} = \text{mg. per 100 cc.}$$

E. ***Interpretation of Urine Urobilinogen Findings.***

1. *Normal Values:* 0.2-3 mg. in a 24 hour specimen.
2. *Increased in hemolytic jaundice* due to the inability of the liver cells to excrete the increased amount of urobilinogen brought to it by the portal blood stream from the intestine where an increased amount is formed.
3. *Increased or normal in hepatogenous jaundice* depending on the ability of the damaged liver cells to excrete the urobilinogen brought to it by the blood stream.
4. *Decreased or none in obstructive jaundice* due to a decreased amount formed in the intestine.
5. *Increased* after a coronary thrombosis.
6. In severe nephritis there may be no urobilinogen present in the urine when other conditions favor its appearance.

VIII. Watson's Two Hour Urine and Short Fecal Urobilinogen Tests.

A. ***Principle:*** It is the same as for the other urobilinogen tests. The two hour afternoon specimen of urine is used because the greatest excretion of urobilinogen occurs during that period.

B. ***General Considerations.***

1. The reagents must be added in the order given; those for the blank are added in the opposite order of those for the unknown.
2. These methods are not as accurate as the quantitative method for urobilinogen in which the urobilinogen is extracted with the petroleum ether, but the increase of the nonurobilinogen substances is roughly proportional to that of urobilinogen.

he Ehrlich unit includes both the urobilin-gen and nonurobilinogen substances; there-ore, the normals are higher in Ehrlich units han in mg. of urobilinogen.

atients with acute porphyria who are ex-reting porphobilinogen in the urine will give n increased value for urinary urobilinogen y this method.

his test is not applicable to urine containing large amount of albumin or bilirubin, espe-ially if with the latter a green Ehrlich re-ction is encountered. It is recommended hat the quantitative method be applied to a 4 hour specimen in these cases.

hotoelectric Colorimeter Method for rine.

2 hour urine specimen is collected in the fternoon.

. The patient is instructed to empty the bladder at 2 P.M. and to drink 1 glass of water.

. The bladder is again emptied at 4 P.M. and this urine is used for the test.

llow the specimen to come to room tem-erature and measure.

lace 2.5 cc. of urine in a colorimeter tube, dd 2.5 cc. of Ehrlich's reagent, and quickly ix.

mmediately (within 15 seconds) add 5 cc. of saturated aqueous solution of sodium acetate nd mix thoroughly.

repare a blank by placing 2.5 cc. of urine nd 5 cc. of the saturated sodium acetate olution in another colorimeter tube and horoughly mix.

ith constant shaking of the tube, slowly dd 2.5 cc. of Ehrlich's reagent.

et the galvanometer at 100 using this blank nd filter No. 565.

ead the unknown within 5 minutes of the ime of the addition of the sodium acetate olution.

btain the mg. per cent of urobilinogen from he table of values made for Watson's fecal robilinogen method on page 114.

alculation for 2 hour specimen:

$$\text{g.\% urobilinogen} \times 4 \times \frac{\text{urine vol.}}{100} = \text{Ehrlich units}$$

n sample.

hotoelectric Colorimeter Method for eces.

ollow Watson's method for fecal urobilin-gen on page 114 as far as the preliminary test and continue as described above for the 2 hour urine urobilinogen test.

alculation:

$$\text{g.\% urobilinogen} \times \frac{500}{10} \times 4 =$$

Ehrlich's units per 100 gm. of feces.

E. ***Method Using Comparator Block.***

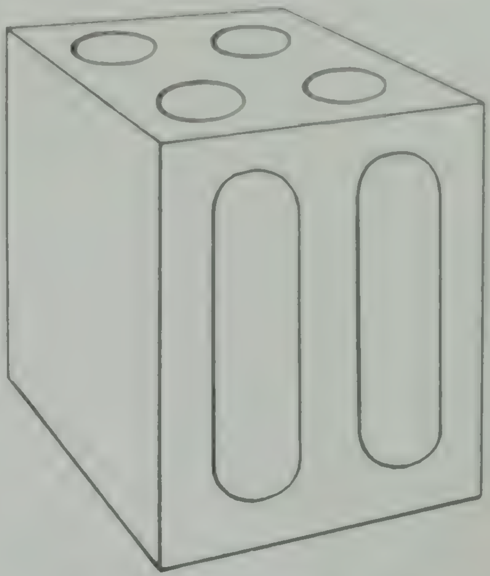


FIG. 13. COMPARATOR BLOCK.

1. A wooden comparator block similar to Fig. 13 is necessary. The openings through the block for color comparison should be 8.5 cm. high and 2 cm. wide. A white piece of paper is placed over the openings in the back.
2. The standards for the comparator block are prepared by pipetting the following amounts of dilute dye solution used in calibrating the photoelectric colorimeter (see page 115) and 0.5% acetic acid into colorimeter tubes.

cc. of dilute dye solution	cc. of 0.5% acetic acid	Equivalent to mg. of dye per 100 cc.	Equivalent to mg. per cent of urobilinogen
0.68	19.32	0.069	0.025
1.35	18.65	0.138	0.05
2.72	17.28	0.277	0.10
4.08	15.92	0.416	0.15
5.44	14.56	0.555	0.20
6.85	13.15	0.699	0.25
8.17	11.83	0.833	0.30
10.90	9.10	1.111	0.40
13.62	6.38	1.389	0.50
16.35	3.65	1.667	0.60

3. Add 1 drop of chloroform to each tube, stopper with a cork, seal by dipping in melted paraffin, and label mg. per cent of urobilinogen.
4. The test is made as described in the photoelectric colorimeter methods (C and D).
5. The blank is placed in one of the front comparator openings and the color standard appearing to match the unknown is placed behind it.
6. The unknown is placed in the remaining rear opening and a tube containing distilled water is placed in front of it.
7. If the color match is not satisfactory, a stronger or weaker dilution is tried until the best match is found.
8. Interpolation may be made if it is evident that

the color intensity lies between two of the standards.

9. Calculate the Ehrlich's units in the same manner as in the photoelectric colorimeter methods.

F. ***Interpretation of the Two Hour Urine and Fecal Urobilinogen Findings.***

1. *Normal Values for Urine:* Up to 1 Ehrlich unit for the two hour specimen of urine. From 1.0 to 1.5 units should be considered doubtful and daily tests done.
2. *Normal Values for Feces:* 130-250 Ehrlich units per 100 gm. Values from 250 to 400 units should be regarded borderline and require serial determinations.
3. For interpretation of abnormal values for urine urobilinogen, see page 116.
4. For interpretation of abnormal values for fecal urobilinogen, see page 116.
   a. If values less than 5 Ehrlich units per 100 gm. are obtained on 4 daily samples, it is believed there is complete exclusion of bile from the intestine.
   b. Values between 5 and 10 Ehrlich units per 100 gm. are regarded borderline, while values over 10 units are believed to indicate that some bile is gaining access to the intestine.

IX. Serum Alkaline Phosphatase.

A. ***Principle:*** Not fully understood. However, it is possible that the liver excretes alkaline phosphatase in the bile.

B. ***Method***—See Section on Chemistry, page 310.

C. ***Interpretation.***

1. Normal in hemolytic jaundice.
2. Greatly increased in obstructive jaundice.
3. Normal or slightly increased in hepatogenous jaundice.
4. Variable in cirrhosis of the liver.

Test for Function of Detoxification

I. Hippuric Acid Test (Quick's Method).

A. ***Principle:*** When benzoic acid is taken into the body, it is combined with glycine by the liver and the resulting compound, hippuric acid, is excreted in the urine. In liver damage the output of hippuric acid is decreased to a degree fairly proportional to the hepatic impairment. The maximum functional capacity is determined since more sodium benzoate is given than can be converted into hippuric acid during the time allowed.

B. ***General Considerations.***

1. Applicable to jaundiced and nonjaundiced patients.
2. Excretion of benzoic acid as hippuric a increases in proportion to body weight.
3. Dehydration, nephritis, hepatorenal diseas or urinary tract obstruction retards the eli nation of synthesized hippuric acid and results in a low output.
4. Passive congestion of the liver in cardiac decompensation results in a low output.
5. In rare instances there may be a toxic reaction to the sodium benzoate.

C. ***Oral Test.***

1. Patient should be instructed to take no drug for 2 days prior to the test.
2. One hour after a light breakfast of toast o cereal and coffee, tea, or milk have the patient empty the bladder completely.
3. Immediately thereafter give orally 6 gm. o sodium benzoate that has been dissolved i 30 cc. of distilled water by heat and the cooled; it may be flavored with a drop o oil of peppermint if desired. Follow with half glass of water. (If patient vomits, the test is of no value.)
4. Have patient save all urine voided during the next 4 hours and to completely empty the bladder exactly at the end of the 4 hou period.
5. If the volume of the urine voided in 4 hours is over 150 cc., acidify with a few drops of acetic acid and evaporate to about 15 cc. (Should the analysis be delayed more than 10 hours, acidify, add a few cc. of toluene to the urine, and place in the refrigerator.)
6. Measure the volume accurately and add 5 gm. of ammonium sulfate for every 10 cc of urine.
7. When the salt has dissolved, filter through a Buchner funnel with filter paper the same size as the filter plate.
8. Add 4 cc. of conc. HCl to the filtrate. Usually this is sufficient to make the urine distinctly blue to Congo red. If not, continue to add conc. HCl until there is a slight excess of acid, i.e., until it turns Congo red paper blue.
9. Stir vigorously until all the hippuric acid is precipitated and allow the specimen to stand in the refrigerator for 1 hour.
10. Filter through a Buchner filter with a Whatman No. 50 filter paper the same size a the filter plate.
11. Wash the precipitate on the filter with small portions of cold distilled water using the washing fluid first to rinse the flask in which the precipitation has been performed.
12. The precipitate is adequately washed when

he washing fluid is free of HCl.

Dissolve the precipitate by passing hot dis-illed water through the filter paper into a lean filter flask.

Titrate this filtrate while hot with 0.2 N NaOH (1 cc. = 0.0358 gm. hippuric acid) using phenolphthalein as an indicator.

Calculation:

a. Number of cc. of NaOH × 0.0358 = gm. of hippuric acid.

b. To this amount of hippuric acid, add the quantity which remains in solution which is 0.1 gm. for each 100 cc. of urine after concentrating.

c. To express hippuric acid in terms of benzoic acid multiply by 0.68.

The washed precipitate may be dried in the air and weighed to the second decimal place on an analytical balance instead of dissolving in hot water and titrating. (Subtract the weight of the filter paper.)

ntravenous Test.

d 2. Same as under oral test.

Have patient drink one glass of water.

nject 20 cc. of sterile sodium benzoate solution intravenously, taking at least 5 minutes or the injection. (The solution contains 1.77 gm. of sodium benzoate in 20 cc. of distilled water and may be obtained from Hynson, Westcott, and Dunning.)

Have patient completely empty the bladder exactly 1 hour after the injection is completed.

Determine the hippuric acid as described in he oral test on only the 1 hour specimen. Do not convert the hippuric acid to benzoic acid because the result is reported as hippuric acid.

nterpretation of the Hippuric Acid Test.

The normal excretion for the oral method s 3 gm. or more of benzoic acid.

a. The normal for persons weighing less than 110 lbs. may be as low as 2.7 gm.

b. There is no upper limit due to increase of excretion with increase in weight.

The normal excretion for the intravenous method is 1 gm. of hippuric acid.

a. The hippuric acid is not converted to benzoic acid as 1 gm. of hippuric acid makes a normal value that is easy to remember.

b. The amount excreted varies with the weight of the patient, a small individual may excrete as little as 0.7 gm. which is the lower limit of normal. There is no upper limit for normal excretion.

The excretion of less than 3 gm. of benzoic acid in the oral test and less than 1 gm. of hippuric acid in the intravenous test in a person of average weight indicates impairment of hepatic function.

4. There is normal excretion in obstructive jaundice without liver cell damage.

Tests for Function of Metabolism

I. Oral Galactose Test (Carbohydrate Metabolism).

A. ***Principle:*** Galactose is rapidly absorbed from the intestine, converted into glycogen by the liver, and stored there temporarily. It has a low renal threshold. When the liver is seriously damaged, this conversion into glycogen is slow or incomplete and the galactose is excreted by the kidneys.

B. ***General Considerations.***

1. Patient should not have any food since the evening before the test and should not eat during the test, but may have water.
2. Applicable to jaundiced and nonjaundiced patients; however, it is not possible to obtain a correct titration for galactose in urine containing bile.
3. Not applicable in diabetic patients that are excreting sugar, in patients with kidney damage, or in diseases of the small intestine.

C. ***Method.***

1. Have patient empty bladder and test urine for sugar.
2. Give by mouth 40 gm. of galactose in 400 cc. of water plus the juice of 1 lemon.
3. Collect urine specimens at hourly intervals for 5 hours and test each specimen for sugar.
4. Pool all specimens containing sugar and measure volume. Discard all negative specimens.
5. Determine the amount of sugar present by Benedict's quantitative method (25 cc. of Benedict's solution is reduced by 0.0625 gm. of galactose).

D. ***Interpretation of the Oral Galactose Test.***

1. Normal excretion in a 5 hour period is 0 to 3 gm.
2. Normal findings are obtained in obstructive and hemolytic jaundice and in localized liver disease.
3. If the liver cells are acutely and diffusely damaged, as in a toxic or an infectious process, there is an increased excretion of galactose.
4. Decreased values are obtained in dehydrated patients and in those with gastric or urinary retention.

II. Intravenous Galactose Test (Bassett, Althauser, and Coltrin's Method).

A. ***Principle:*** Galactose is converted to glycogen by the liver and stored there temporarily. When the liver is damaged, this conversion into glycogen is slow or incomplete and the galactose remains in the blood longer than normal.

B. ***General Considerations.***

1. Patient may have a breakfast of toast and coffee.
2. The test can be used in diabetic patients but not those resistant to insulin.

C. ***Method.***

1. Calculate the amount of solution necessary to inject 1 cc. of a 50% sterile aqueous solution of galactose per kilogram of body weight.
2. Withdraw 5 cc. of blood from a vein and place in a bottle containing an anticoagulant (control).
3. Inject slowly the calculated amount of galactose intravenously with a 100 cc. syringe over a period of 4 to 5 minutes.
4. Obtain 5 cc. of blood at 75 minutes after the injection and place in a bottle containing an anticoagulant.
5. Add 14 cc. of a 20% yeast suspension to 2 cc. of each of the 2 samples of blood and mix thoroughly.
6. Let stand 5 minutes.
7. Add 4 cc. of a tungstic acid solution (prepared fresh by mixing equal volumes of 10% sodium tungstate and 2/3 N sulfuric acid) to each, shake, and let stand a few minutes.
8. Filter and determine the sugar by Folin-Wu's original method using 2 cc. portions, making the final dilution only to 12.5 cc.
9. The following glucose standards are run at the same time making the final dilution only to 12.5 cc.
    - a. 0.5 cc. of 0.1 mg. standard plus 1.5 cc. of distilled water.
    - b. 1 cc. of 0.1 mg. standard plus 1 cc. of distilled water.
    - c. 2 cc. of 0.1 mg. standard.
10. If high values are obtained, dilute the tubes containing the 75 minute blood filtrate to 25 cc. and correct calculation for dilution.
11. *Calculation for Galactose.*
    - a. Subtract the nonfermentable reducing substance in the control blood from the amount in the 75 minute specimen.
    - b. Galactose has a lower reducing power than glucose; so the value obtained in (a) must be increased by 24 per cent.

D. ***Solutions.***

1. *Galactose Solution—50%.*
    - a. The solution must be prepared fresh before each test.
    - b. Dissolve 50 gm. of chemically pure g lactose (Pfanstiehl) in 100 cc. of hot ste triple distilled water.
    - c. Filter through a Buchner funnel with su tion and transfer to a sterile pyrex fla
    - d. Simmer for 10 to 15 minutes, do not a low any of the galactose to dry on th sides or bottom of the container.
    - e. The final volume should be 100 cc., if le than that add enough sterile triple distille water to make 100 cc.
    - f. Cool to room temperature before using
2. *Yeast Preparation.*
    - a. Suspend a weighed amount of fresh Fleishman's yeast in 5 to 10 parts of distilled water, centrifuge, and decant supernatan fluid.
    - b. Repeat until the fluid is clear and colorless and gives no reduction with Benedict's solution (6 to 7 washings).
    - c. Suspend the yeast in 4 parts of distilled water.
    - d. It will keep 2 weeks in the refrigerator
3. Solutions for Folin-Wu's blood sugar determination, see page 266.

E. ***Interpretation of the Intravenous Galactose Test.***

1. Normally no galactose is found in the 7 minute sample of blood.
2. In hepatogenous jaundice the blood value i over 20 mg. per cent.
3. In obstructive jaundice without liver cel damage, it is present but is less than 20 mg per cent.

III. Glucose Tolerance Test (Carbohydrate Metabolism).

A. ***Principle:*** The normal liver rapidly remove any excess of glucose from the blood an stores it as glycogen.

B. ***Method***—See Section on Chemistry, page 26

C. ***Interpretation.***

1. In hepatogenous jaundice the fasting bloo sugar is lower than normal and after the in gestion of glucose the damaged liver cells ar unable to remove it from the blood as rapidl as normal liver cells; so the blood sugar rise higher than normal but returns to norma within 2 hours.
2. In obstructive jaundice the curve rises highe than normal and fails to return to norm until after 2 hours.

IV. Blood Urea (Nitrogen Metabolism).

A. ***Principle:*** Urea is formed in the liver fro the deaminization of proteins.

Method—See Section on Chemistry, page 278.

nterpretation: In extensive damage or de-ruction of the liver the blood urea is low.

Serum Cholesterol and Cholesterol Esters (Lipoid Metabolism).

Principle: The liver removes cholesterol rom the blood, stores it, and also excretes ome in the bile. It causes esterification of holesterol with fatty acids.

Method—See Section on Chemistry, page 288.

Interpretation.

Obstructive jaundice—increase in serum cholesterol and its esters; ratio remains normal.

Degenerative hepatic disease—decrease in serum cholesterol and a greater decrease in esters resulting in a lowered ratio.

Cirrhosis—high serum cholesterol and low esters.

Tests for Regulation of Composition of Blood

I. Prothrombin Time.

Principle: The parenchymal cells of the liver produce prothrombin in the presence of vitamin K.

Method—See Section on Hematology, page 86.

Interpretation.

Prolonged prothrombin time is present in obstructive jaundice. Absence of bile from the intestine prevents absorption of vitamin K. Also prolonged in liver cell damage.

If the prothrombin time is less than 70 per cent after several days of parenteral vitamin K therapy, there is severe liver damage (if other possible causes of a low prothrombin time other than impairment of the liver are ruled out).

II. Fibrinogen Content of Blood.

Principle: Fibrinogen is formed in the liver.

Method—See Section on Chemistry, page 275.

Interpretation: Decreased in liver cell damage.

III. Blood Proteins.

Principle: Liver is the source of serum proteins. Albumin is formed with greater difficulty than globulin in spite of the fact it has a lower molecular weight.

Method—See Section on Chemistry, page 275.

Interpretation.

A decrease in total proteins with a low albumin is found in liver cell damage.

2. In cirrhosis the total proteins may or may not be reduced but there is an inversion of the A/G ratio.
3. In ascites due to cirrhosis there is a marked inversion of the A/G ratio, while little or no change in the A/G ratio occurs in ascites from other causes.
4. Hypoalbuminemia occurs in most cases of obstructive jaundice.

IV. Blood Count for Anemia.

A. *Principle:* The liver stores the antianemic principle and is concerned in its metabolism which results in normal erythrocyte production. It also stores iron.

B. *Method*—See Section on Hematology, page 34.

C. *Interpretation:*

1. Macrocytic anemia with leukopenia.
    a. In severe damage of the parenchyma of the liver of long standing, there may be an average macrocytosis of 8.8 microns or more.
    b. This occurs especially in cirrhosis and Banti's syndrome.
2. There may be a simple iron deficiency hypochromic anemia.

Miscellaneous Tests

I. Cephalin-Cholesterol Flocculation Test (Hanger's Method).

A. *Principle:* Normal serum does not produce a flocculation due to the inhibitory action of albumin on the gamma globulin. A positive flocculation may be due to one or all three of the following:

1. Increase of gamma globulin so that there is an insufficient amount of albumin to inhibit the reaction.
2. Diminution of the serum albumin below the level necessary to inhibit the reaction.
3. Diminution of the flocculation inhibiting properties of the albumin fraction.

B. *General Considerations.*

1. Applicable to jaundiced and nonjaundiced cases.
2. Test must be set up within 4 hours after withdrawing blood from the patient. The blood need not be obtained in a fasting state.
3. Apparatus must be scrupulously clean as a trace of acid or heavy metals will produce a false flocculation.
4. Bacterial contamination of the emulsion will give a false flocculation.

C. *Method.*

1. Pipette 4 cc. of 0.85% NaCl solution into each of 3 conical centrifuge tubes, preferably of the same size and shape.
2. Add to 2 of the tubes 0.2 cc. of the patient's serum; the third tube serves as a control.
3. Add 1 cc. of the diluted cephalin-cholesterol emulsion to all 3 tubes; mix by rotating between the hands.
4. Stopper with cotton and stand in a test tube rack in the dark.
5. Read in 24 and 48 hours.
6. The results are reported according to the character of the precipitate.

Neg. = a milky solution with no visible floccules even when tube is held in front of a light.
+ = a faint precipitate, evenly dispersed, seen clearly only when tube is held up to the light.
+ + = a heavier precipitate than +, but not settling to the bottom of the tube.
+ + + = a heavy precipitate partially settled to the bottom of the tube, supernatant fluid cloudy.
+ + + + = a precipitate completely settled to the bottom of the tube, supernatant fluid clear.

7. The control should always be negative.

D. *Solutions.*

1. *Stock Cephalin-Cholesterol Solution.*
   a. Weigh out 100 mg. of cephalin on an analytical balance and transfer to a bottle which can be stoppered so as to be airtight.
   b. Weigh out 300 mg. of cholesterol and transfer to the bottle containing the cephalin. (The cephalin and cholesterol already mixed may be obtained from the Wilson Laboratories in Chicago or Difco Laboratories in Detroit.)
   c. Add 8 cc. of anesthesia ether, stopper bottle, and rotate to dissolve the cephalin and cholesterol.
   d. This stock solution should be kept in the refrigerator and is good for about 6 weeks.
2. *Dilute Cephalin-Cholesterol Emulsion.*
   a. Pipette 30 cc. of distilled water into a beaker.
   b. Mark the meniscus with a wax pencil and add 5 cc. more of water.
   c. Heat to 60-75°C., remove from flame, and add with stirring 1 cc. of the stock cephalin-cholesterol solution.
   d. Return to flame, stirring continuously, simmer down until the volume is 30 cc. (to the pencil mark made above).
   e. Cool before using.
   f. Will keep 3 days in the refrigerator.
   g. Half amounts of everything can be used to save the stock solution.
3. *Sodium Chloride Solution*—0.85%.

E. *Interpretation of the Cephalin-Cholest Flocculation Test.*

1. *Normal*—no flocculation.
2. No flocculation in 24 hours and a 1 plus 48 hours is considered a negative react
3. A 1 plus in 24 and 48 hours is considere doubtful reaction.
4. The degree of flocculation represents the gree of liver cell damage.
5. The test is negative in obstructive jaund without liver cell damage, in hemolytic ja dice, and in single or circumscribed lesi of the liver.
6. A positive test is obtained in malaria, inf tious hepatitis, and infectious mononucleo

II. Thymol Turbidity and Flocculation T (Neefe's Modification of MacLagan's Method).

A. ***Principle:*** Normal serum does not produ a turbidity in a buffered thymol solution ha ing a pH of 7.55. Serums of patients w liver disease cause a turbidity and precipi tion upon standing. It is thought that t phenolic group has a special affinity for b globulin and lipids under the conditions pr vided and that the turbidity is due to t formation of a globulin-thymol-lipoid co plex.

B. *General Considerations.*

1. Inactivated serum can not be used in the te
2. The units are arbitrary and can not be fined in specific terms.
3. There seems to be a 76 per cent agreeme between the cephalin-cholesterol flocculati test and the thymol turbidity and flocculati test.
4. The blood needs to be obtained in a fasti state.
5. This test can be made less sensitive by usi a buffered thymol solution having a pH 7.8. This less sensitive test when compar with the more sensitive one (pH 7.55) c be used for prognosis in cases of severe liv damage.

C. *Method.*

1. Place 0.1 cc. of serum in a test tube the sa size as those of the artificial standards or in colorimeter tube.
2. Add 6 cc. of the buffered thymol soluti and mix by rotating the tube between t hands.
3. Allow to stand for 30 minutes to 1 hour room temperature and estimate the turbidi by either the artificial standard method or t photoelectric colorimeter method describ below.

After estimating the turbidity, place the tube in a dark place and report the amount of flocculation in 18 hours in the same manner as for the cephalin-cholesterol flocculation test.

Artificial Standard Method.

a. Compare with the artificial turbidity standards against a dark background.
b. Dilute with a measured volume of buffered thymol solution if the turbidity exceeds the 100 mg. per cent standard.
c. *Calculation:*

$$\frac{\text{mg.\% standard}}{10} \times \text{dilution} = \text{units.}$$

Photoelectric Colorimeter Method.

a. Prepare a blank by placing 6 cc. of the buffered thymol solution in a colorimeter tube and adjust the galvanometer to 100 using filter No. 660.
b. Read the unknown and obtain the number of units from the table of values.
c. *Calibration of standard curve.*
    1) Pipette the following amounts of the stock standard barium sulfate solution (shake well between pipetting each amount) and 0.2 N sulfuric acid into a series of colorimeter tubes.

cc. of stock standard solution	cc. of 0.2 N H_2SO_4	Units of thymol turbidity
1.35	8.65	5
2.70	7.30	10
4.05	5.95	15
5.40	4.60	20
6.75	3.25	25
8.10	1.90	30
9.45	0.55	35

    2) Use distilled water as a blank to set the galvanometer at 100 with filter No. 660.
    3) Read each tube immediately after thorough shaking.
    4) Repeat several times using the same stock solution and then repeat with several new stock solutions.
    5) Average the galvanometer readings for each concentration and plot on semilogarithmic graph paper. It should make a straight line.
    6) Make a table of values according to the thymol units for each galvanometer division.

Solutions.

Buffered Thymol Solution—pH 7.55.

a. Place 1.03 gm. of sodium barbital ($NaC_8H_{11}N_2O_3$) in a liter Florence flask.
b. Add 1.38 gm. of barbital ($C_8H_{12}N_2O_3$) and 3 gm. of pure powdered thymol.
c. Add 500 cc. of distilled water and heat just to the boiling point; shake well and cool to room temperature. The solution should be turbid.
d. Add a small amount of powdered thymol crystals and allow to stand overnight at room temperature (20-25°C.).
e. Shake well and then filter the clear solution from the crystalline deposit.
f. Keep in a glass-stoppered bottle at room temperature.
g. It is necessary to test the pH of the solution with a pH meter because different lots of sodium barbital produce a variation in the pH.
h. If the pH of the solution is too alkaline, correct in the following manner:
    1) Make a second thymol solution omitting the sodium barbital; this solution will be acid in reaction.
    2) Add 0.1 cc. portions of the second solution to 10 cc. of the first solution until the correct pH is obtained, checking the pH between each addition with nitrazine paper.
    3) Calculate amount of second solution needed to adjust all of first solution.
    4) Add this amount to the first solution, mix thoroughly, and check the pH with the pH meter.
i. If the solution is too acid, make a new solution using different sodium barbital.

2. *Artificial Turbidity Standards.*
    a. They are the formazin standards of Kingsbury-Clark used for the Kingsbury's test for albumin in urine, see page 8.
    b. They can be made but it is a time consuming procedure.
3. *Stock Standard Barium Sulfate Solution.*
    a. Place 5 cc. of a 0.0962 N barium chloride solution (1% using anhydrous $BaCl_2$) in a 100 cc. volumetric flask and dilute to volume with cold (10°C.) 0.2 N sulfuric acid (1%).
    b. At this temperature the particle size of the precipitated barium sulfate is such that a comparatively stable suspension results.

E. *Interpretation of the Thymol Turbidity and Flocculation Test.*

1. *Normal Values:* 0-5 units of turbidity and either no flocculation or a 1 plus in 18 hours.
2. *Increased in:*
    Parenchymatous liver disease
    Infectious hepatitis
    Cirrhosis
    Weil's disease
    Metastatic neoplasm of liver
    Diabetes (lipemic serum)
    Nephrosis (lipemic serum)

a

Feces

General Consideration

Normal feces consists chiefly of undigested food remnants, various products of digestion, and an enormous number of bacteria, most, if not all, of which are nonpathogenic. In diseases in which digestion is impaired and in those accompanied by diarrhea, food remnants are greatly increased due to passage through the intestine before digestion and absorption are complete. Many of these food remnants can be identified macroscopically, such as seeds, fruit or vegetable skins, etc., or microscopically, such as muscle fibers, vegetable cells, fibers, hair, etc. The quantity of the feces is influenced largely by the amount and character of the food and the amount of water present. A vegetable diet increases the amount. The normal quantity for an adult is around 200 grams a day.

Macroscopic Examination

I. Color.

A. ***Normal***—light or dark brown, due to urobilin.

B. ***Abnormal.***

1. *Yellow*—due to milk diet, cornmeal, rhubarb, senna, santonin, fats, or unchanged bilirubin.
2. *Green*—due to spinach, calomel, or unchanged biliverdin.
3. *Clay or putty* (acholic)—due to absence of urobilin in obstructive jaundice, excess fat in pancreatic disease, and barium taken for Roentgen ray examinations.
4. *Bright red*—due to bleeding low down in the intestinal tract or to undigested beets or tomatoes.
5. *Dark red or chocolate brown*—may be due to an excess of coffee, cocoa, chocolate, blackberries, or cherries.
6. *Black or tarry*—after taking iron, bismuth suboxide, or charcoal, or due to digested blood.

II. Odor.

A. ***Normal***—due to indole, skatol, and butyric acid.

B. ***Abnormal.***

1. *Extremely foul*—indicates putrefaction due to undigested protein reaching the portion of the bowel inhabited by putrefactive bacteria. Usually associated with an alkaline reaction of the feces.
2. *Putrid*—found in ulcerated and malignant tumors of the lower bowel and in large hemorrhages.
3. *Sour and rancid*—indicates gas formation and fermentation of carbohydrates which were inadequately digested or absorbed, or unabsorbed fatty acids. Found in highly acid feces.

III. Form and Consistency.

A. ***Normal***—soft and formed.

B. ***Abnormal.***

1. Soft and watery stools are found in diarrhea or after the use of cathartics.
2. Excessively hard and scybalous stools are present in constipation.
3. Gaseous (fermentative) stools are usually soft and mushy, and bubbles of gas may be present which become more evident after the stools stand in a warm place for 12 hours.
    a. Excessive carbohydrate fermentation produces this type of stool.
    b. A similar bulky, frothy stool when freshly passed is typical of sprue.
4. Flattened or ribbon-like stools are found in spastic colitis or obstruction in the lower portion of the colon.

IV. Mucus.

A. ***Normally*** very small amounts are present.

B. ***Abnormal Amounts***—See Table 23, page 141.

1. Excessive in irritation or inflammation of the intestinal wall.
    a. When thoroughly mixed, the small intestine is involved, and the mucus may only be detected on mixture of the feces with water.
    b. When not thoroughly mixed or only on the outside, the large intestine is involved.
2. Practically pure mucus may be found in dysentery, intussusception, and ileocolitis.
3. Shreds of mucus appearing as firm irregularly segmented strands are found in mucous co-

litis and are sometimes mistaken for pieces of tapeworm.

Mucus may form casts of the intestine as in mucous colitis.

V. Blood.

. *Gross Blood.*

. Note whether the stool is tarry, reddish, or bright red and if blood is evenly mixed, partially mixed, only on the outside, or mixed with mucus.
   a. The higher the origin of the blood in the gastro-intestinal tract the darker and more thoroughly mixed it will be with the feces.
   b. It may come from the mouth or the nose.

. The most common causes of large amounts of blood in feces are ulcers, esophageal varices in cirrhosis of the liver, carcinoma of the gastro-intestinal tract, and hemorrhoids.

. *Occult Blood* (minute traces)—is only detectable by chemical tests.

VI. Parasites.

. *Artifacts.*

. Poorly masticated celery or greens may suggest round worms.

. Cells from oranges suggest pinworms.

. Fibers from banana suggest tapeworms and ova.

. *Parasites.*

. Tapeworms are common.
   a. For identification of segments see Cestodes, page 135.
   b. To examine for tapeworm heads see Cestodes, page 135.

. Some round worms (e.g., Ascaris lumbricoides) are large and easily seen, see Fig. 14, page 130.

3. Hookworms, pinworms, whipworms, and trichinella are small.
   a. When a few are present, they may only be found after mixing the feces with water and running through a fine sieve or gauze.
   b. A hand lens may be needed to identify the smallest ones.

Chemical Examination

I. Fecal Emulsion.

With a wooden applicator take several different portions of the stool and make an emulsion in a test tube three-fourths full of distilled water.

II. Blood.

A. Patient must be on a meat-free diet for 3 days before the test.

B. ***Benzidine Test.***

1. For principle and general considerations see benzidine test for urine on page 14.
2. *Test Tube Method.*
   a. Place a pocket-knife point full of benzidine in a test tube.
   b. Add 3 cc. of glacial acetic acid and shake until the acetic acid is saturated. If necessary add more benzidine.
   c. Allow the benzidine to settle and pour the clear supernatant liquid into another test tube. (A saturated solution, 4 gm. of benzidine in 100 cc. of glacial acetic acid, will keep 2 weeks in a brown bottle.)
   d. To 5 cc. of fecal emulsion in another test tube, add 1 drop of 10% NaOH and 5 cc. of ether.
   e. Shake gently and after the ether separates discard it.
   f. Add 3 drops of glacial acetic acid, mix, and pour 2 cc. of this emulsion into 3 cc. of the benzidine solution.
   g. Add 1 cc. of fresh hydrogen peroxide and mix.
   h. A green to blue color appearing within 5 minutes indicates the presence of blood.
   i. If positive, report as follows:

Trace = faint green
+ = green
++ = greenish blue
+++ = blue
++++ = deep blue

3. *Filter Paper Method.*
   a. Spread a thin layer of feces on a piece of filter paper.
   b. Add 2 drops of the saturated glacial acetic acid solution of benzidine and 1 drop of hydrogen peroxide to the feces.
   c. If a green or blue color appears, the test is positive for blood.

C. ***Guaiac Test.***

1. *Test Tube Method.*
   a. Place a pocket-knife point full of powdered guaiac in a test tube, add 2 cc. of 95% alcohol and mix. (A 1:25 solution of guaiac in alcohol will keep 8 months in a brown bottle.)
   b. Add 2 cc. of fresh hydrogen peroxide and shake.
   c. To 5 cc. of fecal emulsion in another test tube, add 1 drop of 10% NaOH and 5 cc. of ether.
   d. Shake gently and after the ether separates, discard it.
   e. Add 1 cc. of glacial acetic acid and mix thoroughly.
   f. Pour the guaiac solution slowly down the

side of the tube to form a layer on top of the fecal emulsion.
  g. If blood is present, a green to blue color will appear at the zone of contact.
  h. The intensity of color and width of the zone will vary with the amount of blood present. See benzidine test for method of reporting results.

2. *Filter Paper Method.*
  a. Spread a thin layer of feces on a piece of filter paper.
  b. To the feces add a drop of glacial acetic acid, a drop of saturated alcoholic solution of guaiac, and a drop of fresh hydrogen peroxide.
  c. A green or blue color indicates a positive reaction.

III. Bile Pigments.

A. *Bilirubin* (Modified Gmelin's Test).

1. Place 5 cc. of the fecal emulsion in a test tube, add 5 cc. of 10% barium chloride, mix, and let stand a few minutes before filtering.
2. Allow the filter paper to partially dry and then add a drop of yellow nitric acid to it.
3. A positive reaction is indicated by a play of colors, green on the periphery, then in order toward the center, blue, violet, red, and yellow. The absence of green excludes the presence of bile pigment.
4. Fouchet's reagent may be used instead of nitric acid. A blue to green color indicates a positive reaction.
5. Normally unaltered bile pigment is never found in the feces.
6. In diarrheal stools unaltered bile may be present.

B. *Urobilin.*

1. *Schmidt's Qualitative Test.*
  a. Place a few grams of fresh feces in a mortar and add an equal quantity of 10% mercuric chloride solution.
  b. Mix well with a pestle.
  c. Transfer to a shallow evaporating dish and allow to stand 6 to 24 hours.
  d. Urobilin which is normally present will give a red color.
  e. Bile which is not normally present in feces will give a green color.
2. *Quantitative Test*—see tests for fecal urobilinogen in Section on Liver Function Tests.
3. *Interpretation*—See Watson's method for fecal urobilinogen in the Section on Liver Function Tests, page 114.

IV. Reaction.

A. *Nitrazine Paper.*

1. Place a drop of fecal emulsion on a piece of nitrazine paper.
2. After 1 minute compare with the color chart to determine the pH.

B. *Alizarin Test.*

1. Place a small drop of 1% aqueous solution of alizarin on each end of a glass slide.
2. Dip an inoculating wire loop into the feces and mix thoroughly in one of the drops, using the other drop as a color control.
3. An alkaline reaction is indicated by a reddish violet color; neutral, no change; acid, a light yellow color.
4. The density of the colors will depend upon the amount of acid or alkali present.

C. *Normal:* Slightly acid, neutral, or slightly alkaline.

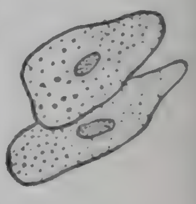
1. Strongly acid stools indicate an excess of carbohydrate in the diet.
2. Strongly alkaline stools indicate an excess of protein.

Microscopic Examination

Place a drop of fecal emulsion on a slide and cover with cover glass. Examine it with low and high power objectives of microscope. Report findings in same manner as reporting urine sediments.

I. Body Cells.

A. *Epithelial Cells.*



1. A few epithelial cells from the wall of the alimentary canal are found normally.
2. Squamous cells come from the anal orifice.
3. They show all stages of disintegration.
4. An excess is found in inflammation of the bowel.

B. *Macrophages.*

1. Macrophages are large mononuclear phagocytic cells with large vesicular nuclei.
2. They frequently contain remnants of ingested leukocytes and erythrocytes.
3. They may be mistaken for endamoeba but can be differentiated by lack of motility and by the character of the nuclei.
4. They show various degrees of necrosis and may appear as outlines of cells or "ghost cells."
5. See Table 23, page 141 for significance.

C. *Leukocytes.*

1. Pus cells are best seen when 1 or 2 drops of 10% acetic acid are added to a drop of fecal emulsion on a slide.

A few are normally present.

An excess occurs in bacillary dysentery, other inflammatory states, and in ulcerative conditions. (See Table 23 on p. 141.)

The number of pus cells roughly corresponds to the extent and severity of the dysentery except in amoebiasis.

Eosinophils may be found in the mucus of the feces of patients with intestinal allergy.

Erythrocytes.

Erythrocytes do not occur normally in feces.

Only found when the lesion is in the colon, rectum, or anus.

It has been stated that they have a tendency to clump in amoebiasis.

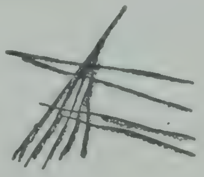
II. Crystals.

Normal.

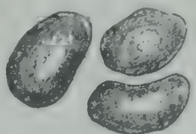
Triple phosphate and calcium oxalate crystals are common after ingestion of certain foods, such as spinach, rhubarb, and some berries. (See drawings on pages 20 and 21.)

Lipoid.

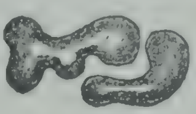
a. Crystals of fatty acids and soaps are common after fatty meals.



1) *Fatty acid crystals* appear as sheaves of large needles or short delicate curved needles which may occur in such thick masses that the shape of the individual crystals can not be made out. When heated they form globules.



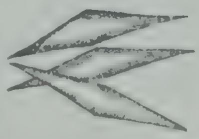
2) *Soaps* occur as short plump crystals or scales.



b. *Neutral fat* and mineral oil appear as round or irregular highly refractive globules.

B. *Abnormal.*

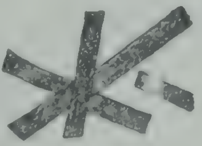
1. *Charcot-Leyden Crystals.*



a. The crystals are colorless, pointed, often needle-like.

b. They are found in ulcerative conditions of the intestine, especially amoebiasis. See Table 23 on page 141.

2. *Hematoidin Crystals.*



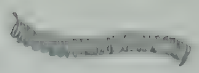
a. The crystals are yellowish or brown needles or rhombic forms.

b. They occur after intestinal hemorrhage.

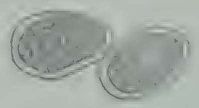
III. Remnants of Undigested Food.

A. *Unstained.*

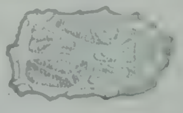
1. *Vegetable Matter.*



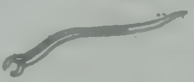
a. *Vegetable spirals* or fibers occur in spirals or tubes having reticulated markings and come from the veins of leafy vegetables.



b. *Vegetable cells* have thick double contoured cellulose shells and contain chlorophyll bodies. They may be confused with parasite ova.



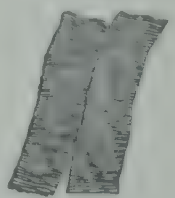
c. *The cellulose framework* of vegetable tissue has a honeycomb appearance.



d. *Vegetable hairs* have a homogeneous and highly refractive wall with a distinct central canal which extends the whole length of the hair. They may resemble larvae of some worms.

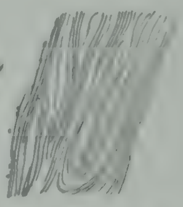
2. *Animal Matter.*

a. *Muscle fibers* appear in varying sizes and shapes.



1) The smaller pieces are irregular or oval and are colored yellow showing they have been partly digested.

2) The large pieces have parallel sides and irregular surfaces and may show transverse and longitudinal striations. These are undigested meat fibers.



b. *Connective tissue* consists of colorless or yellowish threads, with poorly defined edges and indefinite longitudinal striations. They swell and become gelatinous when a few drops of glacial acetic acid are added.

c. *Elastic tissue* generally accompanies connective tissue. Its outlines are more definite, the fibers branch and do not swell but become more distinct in acetic acid.

B. *Stained.*

1. *Starch.*

a. Add a drop of Lugol's solution to a drop of the fecal emulsion on a slide and look for starch under the low power objective.

b. Undigested starch stains blue, partly digested, red.

2. *Fat.*

a. To another drop of fecal emulsion on a slide, add a drop of saturated Sudan III or Scarlet R (Sudan IV) in 70% alcohol and look for fat under the low power objective of the microscope.

b. Neutral fat appears as orange-red droplets, occasionally yellowish flakes.

c. Fatty acid crystals occur in sheaves of large needles or short delicate curved needles which do not stain, but occasionally appear as yellowish flakes.
d. Soaps occur as short plump crystals that do not stain, or as yellowish round or irregular opaque masses.
e. Mineral oil does not take the stain.

IV. Bacteria.

A. ***Bacteria*** constitute about one third of the weight of the dried feces.

B. ***Stained Smear.***

1. Make a thin smear of the emulsion, let it dry, then fix it by passing through a flame, and stain by Gram's method.
2. Normally in adults the predominating bacteria are gram-negative as a great majority belong to the Escherichia (coli) group; there should not be over 5% gram-positive organisms present.
3. The gram-positive organisms may be increased with a high carbohydrate diet.
4. A high percentage of gram-positive organisms is suggestive of intestinal ulceration.
5. Large numbers of Boas-Oppler bacilli may be present in carcinoma of the stomach.
6. In feces of infants a higher percentage of gram-positive organisms is normal due to a milk diet.

C. ***Tubercle Bacilli in Feces.***

1. It is very important to pick whitish, grayish, or blood-stained flakes of mucus or purulent particles to make a smear. In cases of rectal ulcers, swabs of the ulcers are used to make smears.
2. Dry the smear and fix with heat.
3. Stain for tubercle bacilli by Ziehl-Neelsen method, decolorizing with 5% nitric acid in alcohol instead of the usual acid alcohol.
4. If negative, mix the feces with 10 volumes of distilled water and allow to stand for one hour or more.
5. The tubercle bacilli often rise to the surface and collect in the scum.
6. Make a smear from this scum on a slide and stain for tubercle bacilli.
7. If still negative, strain a portion of the above fecal mixture through several layers of gauze to eliminate the larger particles and digest with an equal amount of 2% NaOH.
8. Mix and incubate at 37°C. for 30 minutes.
9. Add 2 to 3 drops of bromthymol blue indicator and 5% HCl until the indicator turns greenish yellow. (Approximately the same amount as the 2% NaOH added above.)
10. Centrifuge at high speed for 30 minutes a pour off the supernatant fluid.
11. Make a smear from the sediment and s for tubercle bacilli.
12. By diluting the sediment with sterile 0.8 NaCl solution, 2 cc. may be injected su cutaneously into the groin of a guinea p for a biologic test.

D. ***Culture of Feces***—See Section on Bacterio ogy, page 179.

V. Yeasts and Molds.

A. ***Yeasts*** are common in feces, especially Blasto cystis hominis.

1. Blastocysts are colorless, refractive, oval, o spherical bodies, 5 to 40 microns in diamete sometimes in the process of division givin them an hour-glass appearance.
2. Most of them have a large clear central bod surrounded by a narrow rim of cytoplas containing a number of refractive spots o nuclei.
3. Some contain round, less refractive masse roughly resembling nuclei and filling the en tire body of the organism.
4. The whole is surrounded by a delicate cap sule and is often mistaken for an amoeba cys
5. See Fig. 17, page 143.

B. ***Molds*** are rare and are usually a contamina tion from unclean vessels or from the air.

1. If pathogenic molds are present, they ma be isolated by making a series of streaks o plates of Sabouraud's or Littman's mediu and incubating at both room temperature an 37°C.
2. See Section on Mycology, page 204 fo identification.

Parasitology

Helminths

I. Nematodes (Roundworms).

A. ***Examination for Nematodes.***

1. *Adult Forms.*
   a. Ascaris lumbricoides is large and can b seen macroscopically.
   b. The other nematodes are very small, a though most of them can be seen macro scopically with careful examination.
   c. Strongyloides stercoralis and Trichinell spiralis are so small that they can only b detected easily with a hand lens.
   d. For description of the adult forms of var ous nematodes see Table 20 and Fig. 14.

TABLE 19. LIFE CYCLE OF THE NEMATODES (Roundworms)

	Ascaris lumbricoides	Necator americanus	Enterobius vermicularis	Trichuris trichiura	Strongyloides stercoralis	Trichinella spiralis
finitive host	Man and dog.	Man.	Man.	Man, hog, monkey, and lemur.	Man.	Man, hog, and carnivorous mammals.
ge of development on leaving st	Unsegmented ovum.	Incomplete embryonated ovum.	Unsegmented or embryonated ovum.	Unsegmented ovum.	Rhabditiform larva.	Larva encysted in muscle.
velopment side of host	No intermediate host. Larva develops in ovum within 10 to 15 days.	No intermediate host. Embryo in ovum develops into rhabditiform larva within 24 hours, then into the filariform larva.	No intermediate host. Larva develops in ovum within very short time.	No intermediate host. Larva develops in ovum within 20 to 40 days.	No intermediate host. Rhabditiform larva may develop directly into filariform larva or into free-living male and female adults which in turn reproduce rhabditiform larva.	Intermediate host: man, hog, and carnivorous animals.
rtal of entry into finitive host	Mouth.	Skin.	Mouth.	Mouth.	Skin.	Mouth.
evelopment in finitive host	↓ Intestine ↓ Blood ↓ Lungs ↓ Trachea ↓ Glottis ↓ Esophagus ↓	↓ Blood ↓ Lungs ↓ Trachea ↓ Glottis ↓ Esophagus ↓	↓	↓	↓ Blood ↓ Lungs ↓ Trachea ↓ Glottis ↓ Esophagus ↓	↓
abitat of ult worm	Small intestine.	Small intestine.	Large intestine and skin of anus.	Lower ileum, cecum, and colon.	Small intestine.	Small intestine.
ngth of life cle (weeks)	8	6	7	9	3	3

Ova and Larval Forms.

a. Mix a small portion of the feces with a drop of water on a glass slide and cover with a cover glass.

b. Examine at least 3 preparations made from different portions of the stool under the low power of the microscope.

c. For description of the ova or larval forms of various nematodes see Table 20 and Fig. 14.

d. If no ova are found, use the concentration method described on page 136.

. ***Life Cycle of the Nematodes***—See Table 19.

. ***Ascaris lumbricoides* (Common Roundworm).**

. For description of adult, ovum, and larva see Table 20 and Fig 14.

. *Fertilized ova* are oval or round averaging 60 by 45 microns. The ovum is covered by an irregular albuminous capsule usually colored brown by bile pigment. Within this capsule is a thick, smooth, double-contoured shell. The embryo is round or oval, usually in the one-cell stage, and is separated from the ends of the shell by a clear space.

3. *Unfertilized ova* are long, oval, often with one flattened side or flattened ends, averaging 75 by 45 microns. The albuminous capsule is coarsely lobulated. The shell is thin and the ovum is completely filled with vacuoles in an unorganized stroma.

D. *Necator americanus* (Hookworm).

1. For description of the adult, ovum, and larva see Table 20 and Fig. 14.

2. *Ova* are broadly oval with a tendency to flattening of the ends, averaging 60 by 40 microns. The shell is smooth, thin, with a single contour. The granular, oval embryo varies from a single-cell stage to an eight-cell stage.

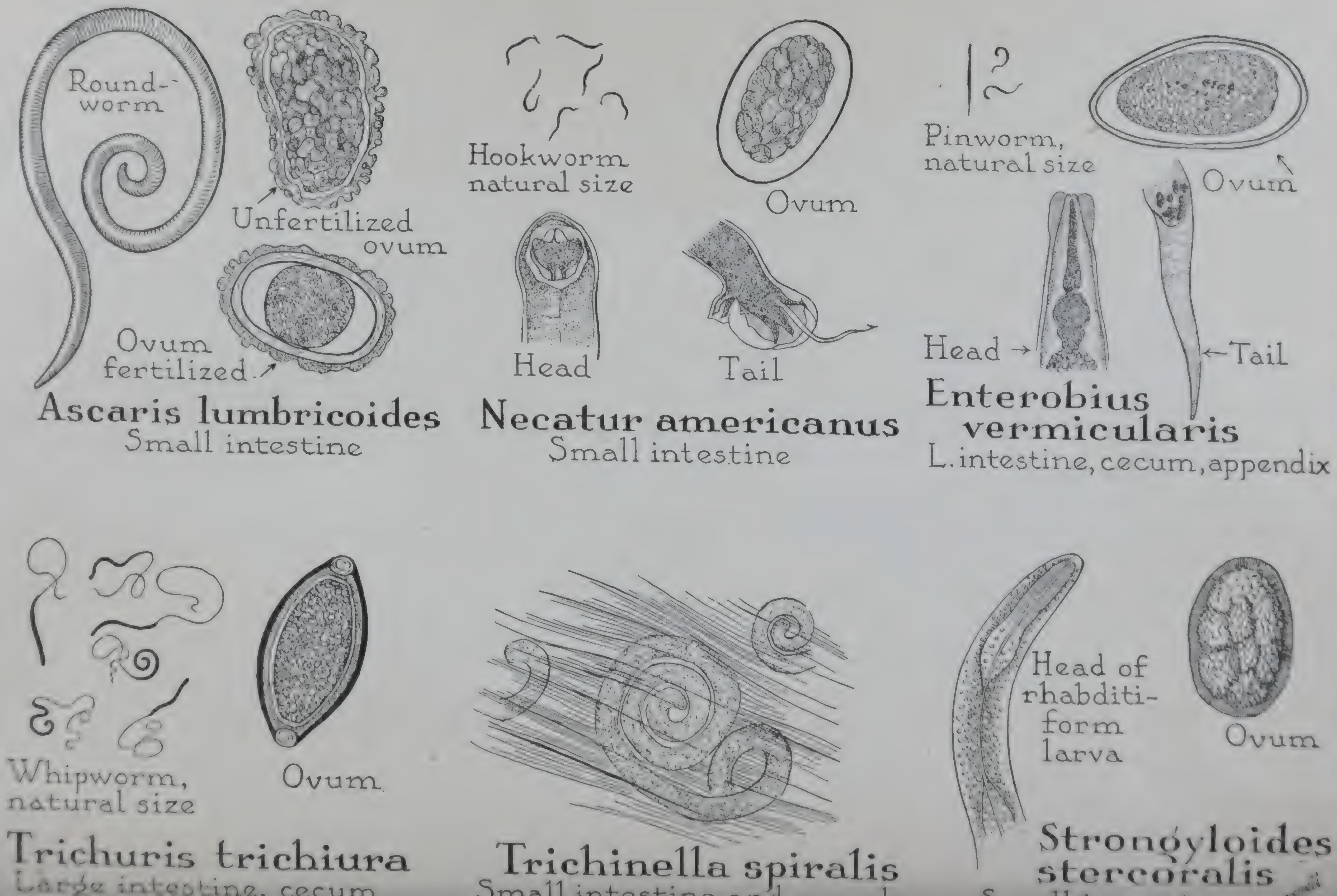

Round-worm
Unfertilized ovum
Ovum fertilized
Ascaris lumbricoides
Small intestine
Hookworm natural size
Ovum
Head
Tail
Necatur americanus
Small intestine
Pinworm, natural size
Ovum
Head →
←Tail
Enterobius vermicularis
L. intestine, cecum, appendix
Whipworm, natural size
Ovum
Trichuris trichiura
Large intestine, cecum
Trichinella spiralis
Head of rhabditiform larva
Ovum
Strongyloides stercoralis

TABLE 20. DIFFERENTIAL CHARACTERISTICS OF NEMATODES (Roundworms)

	Ascaris lumbricoides	Necator americanus	Enterobius vermicularis	Trichuris trichiura	Strongyloides stercoralis	Trichinella spiralis
Ovum						
Size (microns)	45-70 x 35-50	56-60 x 34-40	50-60 x 20-32	50-54 x 22	50-58 x 30-34	No ovum, larva produced viviparously.
Shape	Broadly ovoid.	Ovoid with bluntly rounded ends.	One side convex, other side flattened.	Football-shaped with polar plugs.	Ovoid with bluntly rounded ends.	
Shell	Knoblike, brown outer covering. Thick transparent shell.	Smooth, thin, transparent.	Smooth, thin, transparent.	Smooth, thin, transparent.	Smooth, thin, transparent.	
Rhabditiform larva						
Size (microns)	250 x 14	275 x 16	145 x 10	125 x 8	225 x 16	100 x 6
Morphology	Posterior bulbar swelling of cylindrical esophagus.	Posterior bulbar swelling of esophagus; long, narrow pre-esophageal chamber.*	Posterior bulbar swelling of esophagus.	Slight posterior bulbar swelling of cylindrical esophagus.	Posterior bulbar swelling of esophagus, short pre-esophageal chamber.	Long, narrow esophagus; body coiled.
Filariform larva						
Size (microns)		850 x 35			700 x 28	
Morphology		Sharply pointed tail.			Minute fork at tail.	
Adult						
Female					(Parasitic)	
Length	20-35 cm.	9-11 mm.	8-13 mm.	35-50 mm.	2.2 mm.	3-4 mm.
Diameter	4-6 mm.	0.35 mm.	0.4 mm.	1.7 mm. (Club-shaped posterior end.)	0.04 mm. (Free-living) 1.0 x 0.06 mm.	0.06 mm.
Male						
Length	15-31 cm.	5-9 mm.	2-5 mm.	30-45 mm.	0.7 mm.	1.4-1.5 mm.
Diameter	2-4 mm. (Curved somewhat ventral at the posterior end.)	0.3 mm. (Umbrella-like caudal bursa.)	0.15 mm.	1.3 mm. (Fleshy posterior end coiled like a watch spring.)	0.04 mm.	0.04 mm.
Distinguishing points	3 papillate finely toothed lips, 1 median, 2 ventrolateral of buccal cavity.	A dorsal and ventral pair of cutting plates in the buccal cavity.	A thin, transversely striated cuticular expansion at each side of head.	Anterior 2/3 is a delicate whip-like structure; the posterior 1/3 fleshy.	The cuticula is delicately striated. The worm has a slender, tapering anterior end and a short, conical pointed tail.	Mouth is small orbicular and nonpapillate.

*May be hatched from the ova in the laboratory in 24 to 48 hours.

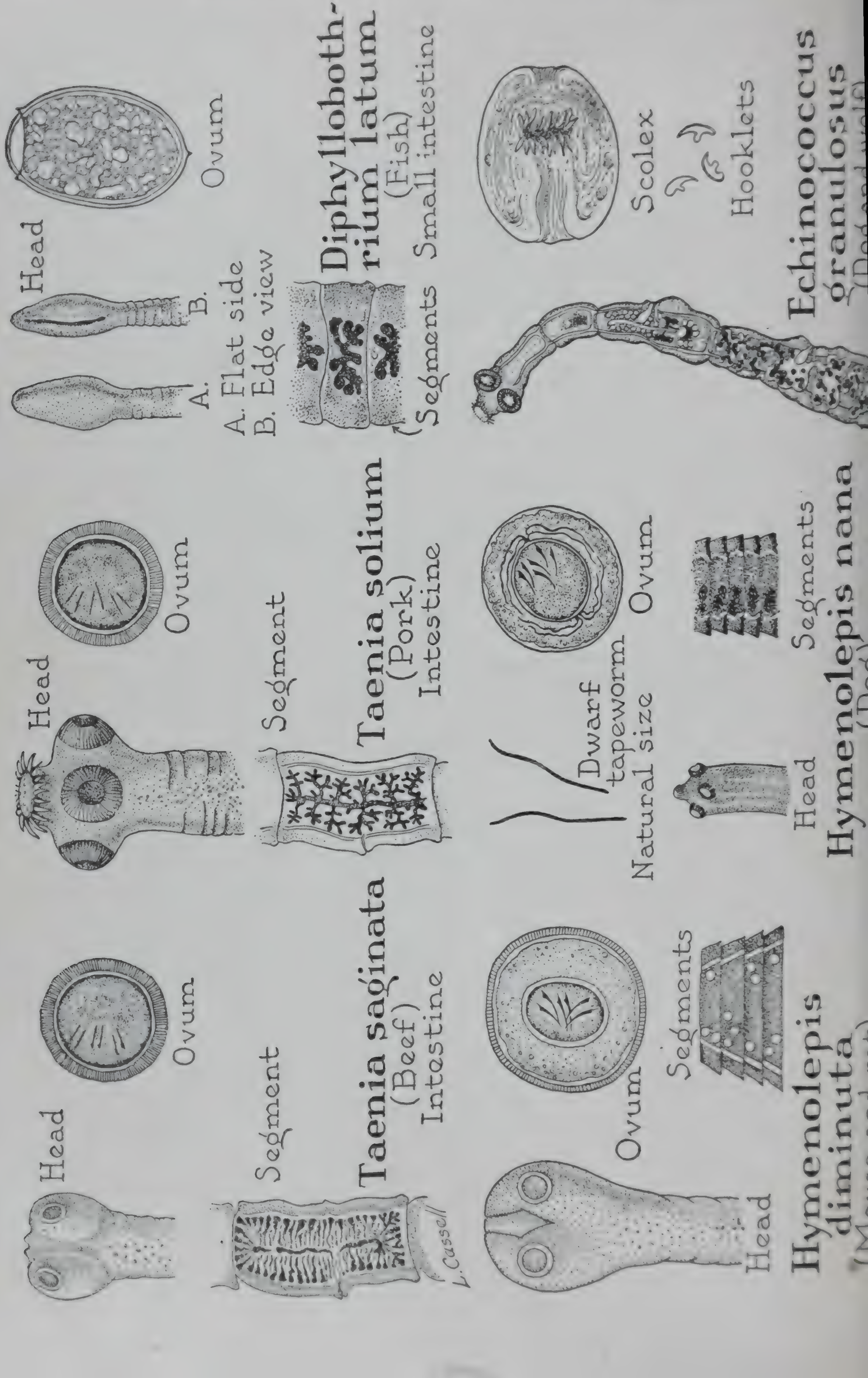

Head
Segment
Ovum
Taenia saginata
(Beef)
Intestine
L. Cassell
Head
Segment
Ovum
Taenia solium
(Pork)
Intestine
Head
A.
B.
A. Flat side
B. Edge view
Segments
Ovum
Diphylloboth-
rium latum
(Fish)
Small intestine
Head
Ovum
Segments
Hymenolepis
diminuta
Natural size
Dwarf
tapeworm
Head
Ovum
Segments
Hymenolepis nana
Scolex
Hooklets
Echinococcus
granulosus

TABLE 21. DIFFERENTIAL CHARACTERISTICS OF CESTODES (Tapeworms)

	Taenia saginata	Taenia solium	Diphyllobothrium latum	Hymenolepis nana	Hymenolepis diminuta	Echinococcus granulosus
Adult worm						
Length	4-8 meters.	2-4 meters.	3-10 meters.	0.5-4.5 cm.	10-60 cm.	3-8.5 mm.
Scolex						
Size (mm.)	1-2 diameter.	0.8-1 diameter.	1 by 2-3 (flat, almond-shaped).	0.3 diameter.	0.2-0.4 diameter.	0.3 diameter.
Suckers	4	4	2	4	4	4
Hooklets	None.	25-30	None.	24-30	None.	30-36
Rostellum	None.	Present.	None.	Present.	Present.	Present.
Proglottides						
Number	1000-2000	800-1000	3000-4000	Up to 200	800-1000	3
Mature	Longer than broad.	Longer than broad.	Broader than long.	Broader than long.	Broader than long.	Longer than broad.
Uterus	15-30 lateral branches.	7-12 lateral branches.	Central, rosette-shaped.	Irregular sac-like.	Irregular sac-like.	Loose coil in terminal proglottid.
Genital pore	Lateral, alternate sides irregularly.	Lateral, alternate sides irregularly.	Middle of flat surface.	Lateral, on same side.	Lateral, on same side.	Lateral.
Ovum (size in microns)	30-40 by 20-30. Yellow to brown with a thick radially striated embryonic shell.	35 by 35. Indistinguishable from T. saginata.	70 by 45. Has a thin double contoured shell with a lid (operculum) at one end which may or may not be open.	Outer membrane 47 by 37. Inner membrane 22 by 28 with gelatinous substance between. Inner membrane has 2 polar thickenings from which arise 4 to 8 slender filaments. Three pairs of hooklets in center of granular mass.	58 by 86. Has a thick yellow outer and colorless inner membrane with a granular intermediate layer. Has no filaments from inner membrane. Three pairs of hooklets present.	Indistinguishable from T. saginata.
Infective larval form	Cysticercus	Cysticercus.	Plerocercoid.	Cercocystis (see text).	Cercocystis.	Echinococcus.
Host						
Definitive	Man.	Man.	Man, dog, cat, and other mammals.	Rats, mice, and man.	Rats, mice, man, and dog.	Dog and wolves.
Intermediate	Cattle.	Hog and man.	Copepod, then fish.	None.	Fleas, beetles, cockroaches, etc.	Man and herbivorous animals such as sheep, horses, hogs, etc.
Stage in Man	Adult.	Adult or larval.	Adult.	Adult or larval.	Adult.	Larval.
Location in Man	Small intestine.	Small intestine.	Small intestine.	Small intestine.	Small intestine.	Various organs.

It is separated from the shell by a clear space.

3. In stools two or more days old the larvae may be fully developed as tiny folded or coiled worms within the eggs or may be free in the feces.

E. ***Enterobius vermicularis* (Pinworm).**

1. For description of adult, ovum, and larva see Table 20 and Fig. 14.
2. *Ova.*
    - a. Ova are oval with one side flat and the ends slender averaging 55 by 26 microns.
    - b. The shell is smooth with a double contour.
    - c. The embryo is separated from the shell by a clear space.
    - d. The larva may be partly or fully developed.
3. *Examination for Ova and Larvae.*
    - a. Ova and larvae are found in the feces in only 5 to 10 per cent of infected persons. They are usually on the outer surface of the stool.
    - b. Sometimes ova or larvae may be found in the urine of female patients.
    - c. The ova and larvae are usually found in scrapings from the perianal region by using either a NIH or Graham swab (for description of swabs see g and h).
    - d. Gently but firmly pass one of the swabs over the perianal region, preferably in the morning before the patient has defecated.
    - e. The cellophane or scotch tape is removed and placed on a slide over a drop of water.
    - f. A drop of water is then placed on top and this covered with a cover glass; examine under the low power objective of a microscope.
    - g. *The NIH swab* was developed at the National Institute of Health.
        - 1) Fasten a piece of cellophane 1 inch square over the tip of a glass rod by means of a rubber band.
        - 2) The other end of the glass rod is passed through the hole in a rubber stopper which will fit a test tube.
        - 3) The cellophane tip should not touch the bottom of the test tube.
    - h. *The Graham swab* consists of a piece of scotch tape 2 inches long placed over the butt end of a test tube with the sticky side out.

F. ***Trichuris trichiura* (Whipworm).**

1. For description of adult, ovum, and larva see Table 20 and Fig. 14.
2. *Ova* are slender, oval, averaging 52 by 23 microns, with a round knob at each end. The color is brown and the shell smooth, thick, with a double contour.
3. The embryo within the shell may fill the tire space or may be separated from the at the ends.

G. ***Strongyloides stercoralis.***

1. The *larvae* are found in the feces.
    - a. Their movement is vigorous and like and must be distinguished from h worm larvae.
    - b. For description see Table 20 and Fig.
2. *Rhabditiform larva.*
    - a. Measures 225 by 16 microns.
    - b. The anterior end is rounded, the poste pointed.
    - c. The buccal cavity is short, less than the diameter of the body. (The length the buccal cavity of the hookworm la is the same as the diameter of the body.
    - d. The genital anlage, located just poste to the middle of the body, is a large cell. (Anlage of hookworm larva is sma
3. *Filariform larva.*
    - a. Measures 700 by 28 microns.
    - b. The buccal cavity has disappeared.
    - c. The esophagus is one half the length of larva. (It is one fourth the length in hookworm larva.)
    - d. The genital anlage is not prominent.
    - e. The tail is notched at the tip. (Tail hookworm larva is pointed.)

H. ***Trichinella spiralis.***

1. Infection occurs from eating raw or insuff ently cooked pork which contains encys larvae.
2. The larvae mature to adult forms in the sn intestine in 2 days and reproduce in ab 6 days.
    - a. The new larvae migrate to striated mus by way of the lymphatics and bl stream.
    - b. They attain a maximum length of 0.1 n and assume a characteristic spiral sha
    - c. An ellipsoidal capsule of sarcolemm origin is formed about the coiled wo in 15 to 20 days.
    - d. These encysted larvae may be found tissue removed for biopsy from a mus near its attachment to a tendon.
    - e. For description see Table 20 and Fig.
3. *Skin Test.*
    - a. An infected patient will give a posit skin test within 30 minutes after the int dermal injection of an extract of the l vae.
    - b. The extract is made from larvae obtain by pepsin digestion of muscles of an fected animal by a method similar in pr

ciple to that for pollen extracts described in the Section on Allergy Extracts.

II. Cestodes (Tapeworms).

xamination for Cestodes.

xamination of Proglottids (Segments).

. Place 2 or 3 segments between 2 glass slides and press together.

b. Hold up to the light or place under the low power objective of a microscope and examine the uterus and genital openings.

. For a description of the segments of various tapeworms see Table 21 and Fig. 15.

Examination for Scolex (Head).

a. Patient should have no solid food for 24 hours and a thorough purging with salts.

b. Administer an anthelmintic by duodenal tube, followed in 15 minutes by a large dose of salts through the tube.

c. Collect the following evacuations and examine for the head by passing the liquid stool through a 20 mesh wire sieve.

d. Invert the contents that remain in the sieve in a black-bottomed pan and examine for the head with a hand lens.

e. For identification of the scolex of the various tapeworms see Table 21 and Fig. 15.

Examination for Ova.

a. Mix a small portion of the feces with a drop of water on a glass slide and cover with a cover glass.

b. Examine at least 3 preparations made from different portions of the stool under the low power of the microscope.

c. For a description of the ova of the various tapeworms see Table 21 and Fig. 15.

d. If no ova are found, use the concentration method described on page 136.

Taenia saginata (Beef Tapeworm).

For description of the adult worm and ovum see Table 21 and Fig. 15.

Cattle are infected by ingesting ova which develop into cysticerci (encysted larvae) in the tissue.

When raw or poorly cooked beef containing cysticerci is eaten by man, the larvae may develop into adult worms in the small intestine.

Taenia solium (Pork Tapeworm).

For description of the adult worm and ovum see Table 21 and Fig. 15.

Hogs ingest ova which develop into cysticerci (encysted larvae) in the tissues.

Man is infected by eating raw or poorly cooked pork containing cysticerci.

Man may also develop cysticerci in any tissue in the following manner:

a. Ingestion of food or water contaminated with ova.

b. Oral transmission of ova by unclean hands of carriers of the adult worm.

c. Auto-infection by the regurgitation of ova by reverse peristalsis into the stomach or duodenum where the ova hatch and produce larvae.

d. These larvae invade the tissues and produce cysticerci.

D. *Diphyllobothrium latum* (Fish Tapeworm).

1. For description of the adult worm and ovum see Table 21 and Fig. 15.
2. The ciliated embryo (coracidium) is liberated from the shell of the ovum in water and is ingested by a copepod in which the elongated larva develops.
3. The copepod is ingested by certain fresh water fish in the flesh of which the larva undergoes further development into a plerocercoid larva.
4. Man is infected by eating raw fish containing the larvae.

E. *Hymenolepis nana* (Dwarf Tapeworm).

1. For description of the adult worm and ovum see Table 21 and Fig. 15.
2. There is no intermediate host and man becomes infected either directly from hand to mouth or indirectly by food and water contaminated with ova.
3. The oncosphere of the ovum is liberated in the small intestine and penetrates a villus to become a cercocystis.
4. This larva breaks out of the villus into the lumen of the intestine where it develops into an adult worm.
5. Ova may continue this cycle in the intestine instead of being passed into the feces.

F. *Hymenolepis diminuta* (Mouse and Rat Tapeworm).

1. For description of the adult worm and ovum see Table 21 and Fig. 15.
2. The intermediate hosts are the larval and adult forms of certain insects in which the ovum develops into a cercocystis.
3. Man is usually infected from the ingestion of food containing the intermediate hosts.

G. *Echinococcus granulosus* (Dog Tapeworm).

1. For description of the adult worm and ovum see Table 21 and Fig. 15.
2. Man ingests the ova which develop into larvae in the duodenum where they attach their hooklets to the mucosa.

3. The larvae penetrate the intestinal wall, pass into the lymphatics or mesenteric veins, and are carried to various parts of the body.
4. They most frequently enter the portal vein and lodge in the liver.
5. If the larvae are not destroyed by phagocytic cells, they may develop into echinococcal (hydatid) cysts.
6. An echinococcal cyst consists of an inner germinal layer which produces buds or scolices that grow into the cavity of the cyst and an outer protective chitinous layer.
7. The scolices are equipped with suckers and a double crown of hooklets (see Fig. 15).
8. The scolices represent the fully developed larvae and may produce daughter, granddaughter, etc., cysts within the original cyst, or produce new cysts elsewhere in the body if they should break through the capsule of the original or primary cyst.
9. The examination of feces is of no help in the diagnosis of echinococcal cysts.
10. Diagnosis is made by one of the following methods:
    a. *Finding scolices or hooklets* either in fluid obtained from an echinococcal cyst, in sputum after the rupture of a cyst in the lung, or in urine after the rupture of a cyst in the kidney.
    b. *Precipitin test.*
        1) The antigen is undiluted fluid from an echinococcal cyst.
        2) Carefully overlay 0.5 cc. of the patient's serum in a small test tube with an equal quantity of cyst fluid.
        3) Incubate at 37°C. for 30 minutes.
        4) A positive test consists of a white ring at the junction of the 2 fluid layers.
    c. *Skin test.*
        1) The antigen is fluid from an echinococcal cyst which has been filtered through a Seitz filter and preserved with 0.5% phenol.
        2) Inject 0.1 cc. of antigen intradermally in the forearm.
        3) In the opposite arm inject 0.1 cc. of sterile 0.5% phenolized 0.85% NaCl solution as a control.
        4) A positive reaction is a wheal formation with edema and surrounding redness within 30 minutes.

III. Trematodes (Flukes).

A. *Adults.*

1. The adult forms are rarely found except at autopsy. Fasciolopsis buski is occasion found in the feces or vomitus.
2. *Examination of Adult Flukes.*
    a. Place the fluke between two glass s and press the slides together.
    b. Examine by holding slides up to the l or by the low power objective of the croscope.
    c. Sections and stains may be made of adult after fixing in 10% formalin.
    d. For identification see Table 22 and Fig.

B. *Ova.*

1. Fluke infestation is usually diagnosed by fin ing the ova.
2. See Table 22 for the material to examine the ova of the various flukes. Make wet pre arations of the material on slides and cov with a cover glass.
3. The material must be examined soon after o taining it from the patient because the o develop into larvae very quickly.
4. For description of the ova of various fluk see Table 22 and Fig. 16.
5. If no ova are found, concentrate with t centrifugation method described below.

IV. Concentration of Ova and Cysts.

A. If ova or cysts are not found with an emulsi of feces, use one of the following concentr tion methods.

B. *Centrifugation Method.*

1. Mix thoroughly about 10 gm. of feces wi 75 cc. of warm water (about 40°C.).
2. Strain into two 50 cc. centrifuge tub through a sieve (40 mesh to an inch) or layer of wet gauze. This removes any lar coarse particles.
3. Centrifuge for 1 minute at low speed. (T is sufficient length of time to throw only o or larvae to the bottom of the tube.)
    a. The diluted feces consists of a suspensi of particles most of which are much sma er than ova.
    b. These small particles are thrown do more slowly than the larger, heavier o
    c. It is important, therefore, to run the cent fuge just long enough to throw the o to the bottom and to stop before t smaller particles are thrown down.
4. Pour off all the contents of the tube exce the sediment in the bottom which should co tain the ova.
5. Refill the tube with warm water, shake we and centrifuge again.
6. Repeat this several times until the supernata fluid is reasonably clear.

Table 22. Differential Characteristics of Trematodes (Flukes)

	Liver Flukes		Intestinal Fluke	Lung Fluke
	Fasciola hepatica	Clonorchis sinensis	Fasciolopsis buski	Paragonimus westermani
ult				
olor	Brown.	Gray.	Pinkish.	Reddish brown.
ize				
Length	2-3 cm.	11.5-20.1 mm.	2.0-7.5 cm.	0.8-1.6 cm.
Breadth	0.8-1.3 cm.	2.8-4.6 mm.	0.8-2.0 cm.	0.4-0.8 cm.
hape	Leaf-like with cephalic cone.	Elongate.	Ovate.	Changes shape.
ntegument	Scales.	Smooth.	Spines.	Spines.
Ratio of size of oral o ventral sucker	Equal in size.	Oral larger than ventral.	Oral ¼ size of ventral.	Equal in size.
ocation in host	Liver, large and small bile ducts.	Large and small bile ducts.	Duodenum and jejunum.	Cystic cavities in lung tissue.
um				
ize (microns)	130-150 by 63-90.	29-26 by 27-35.	130-140 by 80-85.	73-118 by 46-47.
Shape	Ellipsoidal.	Oval.	Ellipsoidal.	Ellipsoidal.
Operculum	Present.	Present.	Present.	Present.
Exit from host	Feces.	Feces.	Feces.	Sputum and feces.
e cycle				
Adult and Ovum (only important definitive hosts given)	Man, sheep, and cattle.	Man, dog, cat, and hog.	Man and hog.	Man, cat, dog, and hog.
Miracidium	Water.	Snail.	Water.	Water.
Sporocyst and redia	Snail.	Snail.	Snail.	Snail.
Cercaria	Water.	Water.	Water.	Water.
Encysted cercaria	Water plants.	Fish.	Water plants.	Crab and crayfish.
Entry in definitive host	Mouth.	Mouth.	Mouth.	Mouth.

	Blood Flukes		
	Schistosoma haematobium	Schistosoma mansoni	Schistosoma japonicum
lult			
Male			
Size (cm.)			
Length	1.0-1.5	0.6-1.4	0.9-2.2
Breadth	0.09	0.11	0.05
Integument	Finely tuberculated.	Grossly tuberculated.	Minute spines and sucker and gynecophoric canal.
Female			
Size (cm.)			
Length	2.0	1.2-1.6	1.2-2.6
Breadth	0.025	0.016	0.03
Integument	Smooth.	Smooth.	Smooth.
Location in host	Pelvic veins.	Mesenteric veins.	Mesenteric veins.
vum			
Size (microns)	112-170 by 40-73.	114-182 by 45-73.	74-106 by 55-80.
Shape	Spindle (rounded anterior and conical posterior end).	Elongate, oval (sometimes with S-like curve).	Oval to rounded.
Spine	Terminal, delicate with blunt point.	Lateral, long.	Lateral, short, sometimes curved (knoblike).
Location in host	Veins of bladder, occasionally rectum, and pelvic lymph glands.	Veins of colon, rectum, occasionally bladder, and mesenteric lymph glands.	Veins of intestine and rectum.
Exit from host	Urine, rarely feces.	Feces, rarely urine.	Feces.
e Cycle			
Adult and ovum	Man and monkey.	Man and monkey.	Man, dog, cat, cattle, horses, and hogs.
↓ Miracidium	Water.	Water.	Water.
↓ Sporocysts (1 and 2)	Snail.	Snail.	Snail.
↓ Cercaria	Water.	Water.	Water.
↓ Entry to definite host	Skin.	Skin.	Skin.

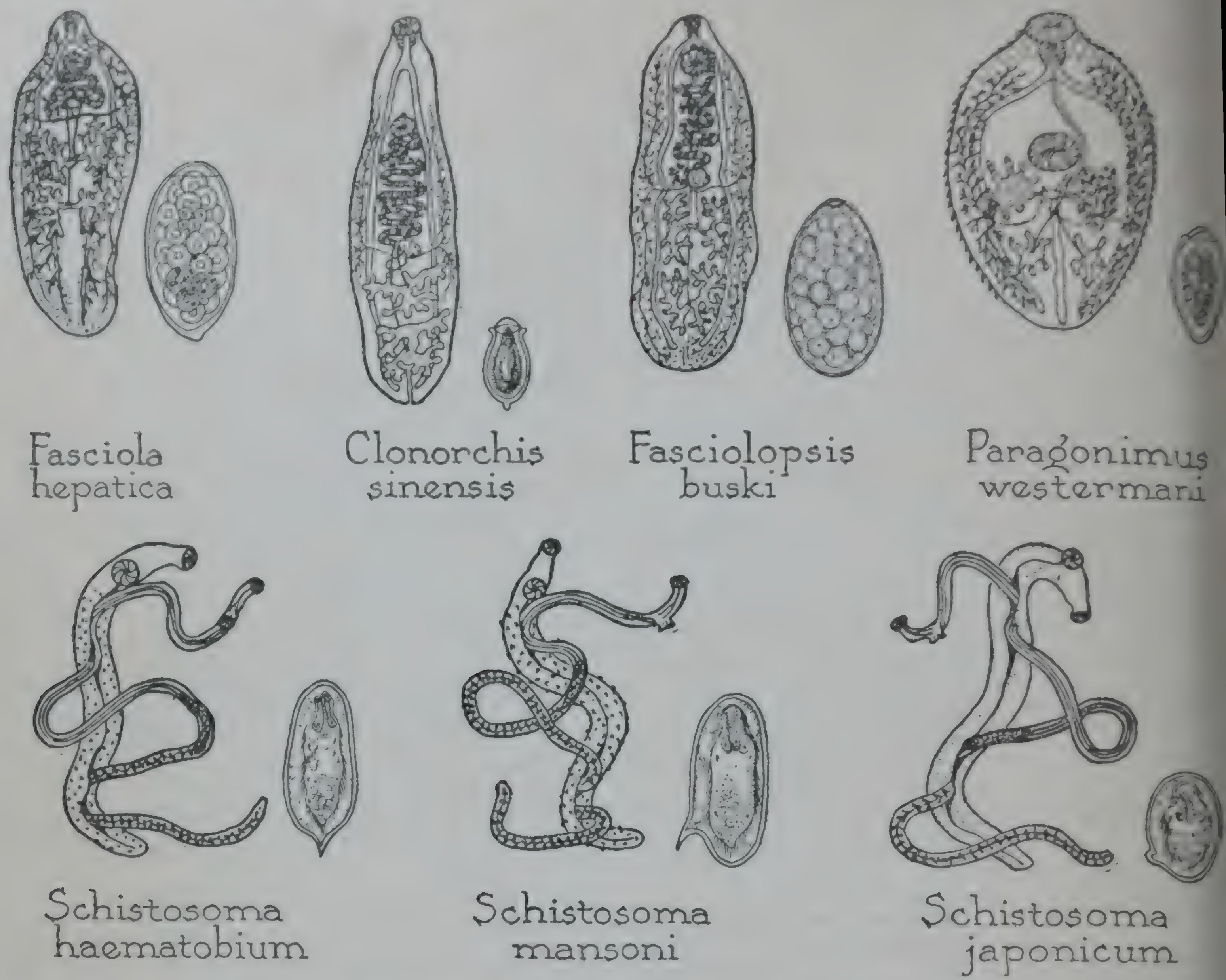


Fig. 16. Trematodes—Flukes. (Reproduced from Belding's "Textbook of Clinical Parasitology," published by D. Appleton-Century Co., New York City.)

7. After pouring off the supernatant fluid for the last time, shake the tube and make several slide preparations of the sediment.
8. Examine for ova and cysts, if none are found proceed with the zinc sulfate flotation method.

C. ***Zinc Sulfate Flotation Method (Faust).***

1. Continue with the 2 tubes used in the centrifugation method by washing the sediment of one tube into the other tube with 33% zinc sulfate solution.
2. If necessary, add more zinc sulfate solution to fill the tube and shake thoroughly.
3. Centrifuge for 1 minute at 1000 revolutions per minute and skim off the surface film with a wire loop for smears.
4. Examine unstained wet preparations for ova and iodine stained preparations as well as hematoxylin stained smears for cysts.

Protozoa

I. Examination for Protozoa.

A. ***Direct Examination for Trophozoites.***

1. *Trophozoites* or motile forms are usually found in liquid or mushy stools.
    a. If a purge is given, the second evacuation is the best one to examine.
    b. At least 3 stools should be examined before a negative report is made.
2. The specimen must be kept warm (body temperature) and examined within 30 minutes after evacuation because the trophozoites die rapidly and disintegrate upon exposure to air.
3. Keep specimen in the 37°C. incubator until examined.
4. Warm the stage of a microscope by placing a substage lamp underneath the diaphragm and enclosing the lower portion of the microscope with a towel.
5. Particles of the bloody purulent mucus, if present, or portions from different parts of the specimen are mixed in a drop of warm 0.85% NaCl solution placed on each end of a warm slide.
6. Cover with a cover glass and examine carefully with a high dry objective of the microscope, reducing the light until hyaline objects are clearly seen.

xamine at least 3 such slides.

xamine iodine stained preparations of all pecimens for cysts.

irect Examination for Cysts.

he *cyst* stage is usually found in formed nd mushy stools which may be examined ny time up to 24 hours after the specimen s passed.

. Trophozoites form cysts only in the intestine.

. Precysts may be found which resemble trophozoites, except they are round like cysts and are not motile.

ake a small portion of the specimen and nix with a drop of D'Antoni's iodine solution on a glass slide.

lace a cover glass over the drop and examine fter 5 minutes.

The cytoplasm of the cyst stains yellow, glycogen a dark red or brown, chromatin almost black. The nuclei are sharply differentiated.

Examine 3 or 4 preparations.

D'Antoni's Iodine Solution.

a. *Standardized solution of 10% KI.*

1) Place 100 gm. of potassium iodide in a liter volumetric flask, add enough distilled water to dissolve, and dilute to volume.
2) Weigh a 25 cc. volumetric flask to 4 decimals and then fill to volume with the KI solution and weigh.
3) Return the KI solution to the original flask.
4) Subtract the weight of the flask from the weight of the flask plus the solution to obtain the actual weight of the solution which theoretically should be 26.9250 gm.
5) Calculate the amount of KI to add to the liter of solution to make it an accurate 10% solution.
6) *Calculation.*

$$x = 10 - \left[\frac{26.9250 - \text{(actual weight)}}{26.9250} \times 100\right]$$

x = actual percentage of solution.

$$\frac{1000}{x} - 100 = \text{gm. of KI to be added to the liter of solution.}$$

7) Add the KI to the solution and mix until dissolved.
8) Again weigh and calculate the actual percentage of the solution which should be between 9.9990 and 10.0000%.
9) Solution keeps well in a tightly stoppered brown bottle.

b. *D'Antoni's solution.*

1) Place 10 cc. of the standardized 10% KI solution in a 100 cc. volumetric flask, add 1.5 gm. of powdered iodine crystals, and make up to volume with distilled water.
2) Keep in a tightly stoppered brown bottle and let stand 4 days before using.
3) For staining, filter about 10 cc. into a tightly stoppered brown bottle.
4) This filtered solution should not be used more than 4 weeks.

C. ***Concentration of Cysts***—See method on page 136.

D. ***Stains for Protozoa.***

1. *Method A.*
   a. Make a thin smear of the feces on a slide.
   b. While the smear is still moist, fix in hot (50 to 60°C.) Schaudinn's solution plus 10% acetic acid for 10 minutes. (For flagellates use 20% acetic acid.)
   c. Alcohol (95%) plus iodine (port wine color) for 5 minutes.
   d. Alcohol (70%) for 5 minutes (may be left in this alcohol an indefinite length of time).
   e. Rinse in running tap water 1-3 minutes.
   f. Ferric alum (4% aqueous solution) for 14 minutes.
   g. Rinse in tap water for 1-2 minutes.
   h. Stain for 10 minutes in 0.5% aqueous solution of hematoxylin made from the stock solution.
   i. Decolorize in 0.25% aqueous solution of ferric alum for 12 minutes (flagellates 8-10 minutes).
   j. Wash in running tap water for 3 to 30 minutes.
   k. Dehydrate for 1 minute in each of the following: 50, 70, 85, 95, and 100% alcohol.
   l. Clear in 2 changes of xylol for 2 minutes each and mount in clarite.
   m. At no stage should the smears be allowed to dry but should drain between solutions.
   n. *Solutions.*
      1) *Schaudinn's solution.*

$HgCl_2$ (Sat. aq. sol.)	2 parts
Alcohol (95%)	1 part

      2) *Stock hematoxylin solution* (10%).

Hematoxylin	10 gm.
Alcohol (absolute)	100 cc.

Allow to ripen for several weeks before using.

2. *Method B.*
   a. Make a thin smear of the feces on a slide and while still moist fix in the tannic acid mixture for 5-7 minutes or indefinitely.
   b. Wash in tap water 3 or 4 times.
   c. Place in ferric alum (4% aqueous solution) for 3 minutes or longer. (If solu-

tion is warmed to 45 or 50°C. leave slides in 1-3 minutes.)

- d. Wash well 3 or 4 times in tap water.
- e. Stain for 3-5 minutes in 0.5% aqueous hematoxylin solution made from a stock solution (see Method A).
- f. Wash in tap water.
- g. Decolorize 30 seconds to 1 minute or longer in a 3% solution of picric acid in 70% alcohol.
- h. Dehydrate for 1 minute in each of the following: 50, 70, 85, 95 and 100% alcohol. (A 70% alcohol with a few crystals of potassium acetate may be used for over decolorized smears; makes stain more bluish.)
- i. Clear for 2-3 minutes in carbol-xylol (1 part phenol to 3 parts xylol) or in benzene and mount in clarite.
- j. At no stage should the smears be allowed to dry but should drain between solutions.
- k. *Tannic acid fixative.*

Tannic acid, c.p.	4 gm.
Ethyl alcohol (70%)	90 cc.
Glacial acetic acid	5 cc.
Phenol crystals melted	1 cc.

E. *Cultures for Protozoa.*

1. *Cleveland and Collier's Medium.*
   - a. Prepare Difco's endamoeba medium according to directions.
     - 1) Place 4 cc. in narrow test tubes.
     - 2) Autoclave 20 minutes at 15 lbs. pressure and slant.
   - b. Add about 2 cc. of a 1 to 6 dilution of human serum in sterile 0.85% NaCl solution to each tube of medium and a tiny speck of sterile rice starch.
   - c. Using a sterile wooden applicator, inoculate the serum-saline portion of the medium with a small portion of the feces and mix thoroughly.
   - d. After 24 and 48 hours incubation at 37°C., examine for protozoa in the following manner:
     - 1) With a capillary pipette remove a small amount of sediment from the bottom of the fluid in the culture tube.
     - 2) Place a drop on each end of a warm glass slide, cover with a cover glass, and examine as described under direct examination for trophozoites.
     - 3) It is often difficult to differentiate E. coli from E. histolytica because E. coli trophozoites become more active in cultures.
2. *Boeck and Drbohlav's Medium.*
   - a. *Preparation of medium.*
     - 1) Wash 4 eggs with tap water, brush with alcohol, and carefully break into a sterile flask containing glass beads.
     - 2) Add 50 cc. of Locke's solution which has been filtered and autoclaved at 121°C. for 15 minutes. (See p. 258 for preparation of Locke's solution.)
     - 3) Shake until the eggs are thoroughly broken up and place 4 cc. in narrow test tubes.
     - 4) Slant the tubes in an inspissator and autoclave in the same manner as Saenz's medium (see p. 179).
   - b. Add enough of a 1 to 9 dilution of serum in sterile Locke's solution to cover the medium.
   - c. Scoop up several large loopfuls of sterile rice starch and add to the medium.
   - d. Using a sterile wooden applicator, inoculate the fluid portion of the medium with a small portion of the feces and mix thoroughly.
   - e. Examine for protozoa after 24 and 48 hours incubation at 37°C. as described under Cleveland and Collier's medium.

II. Endamoebae.

A. *Trophozoite Stage in Amoebiasis.*

1. The disease consists chiefly of ulceration of varying extent in the colon; the trophozoites are present and reproduce by direct division in and on the ulcerated areas.
2. More or less blood and mucus containing the amoebae are being discharged constantly from the ulcers.
3. During the period of acute active disease there may be many stools daily, consisting chiefly of mucus and blood. In the chronic stage the stools may be formed and then it is best to examine the second and third specimens after a saline cathartic.
4. Examine specimens as described above for trophozoites.
5. Endamoeba histolytica appears as a large cell, 20 to 35 microns in diameter, surrounded by a clear ectoplasm and containing coarse granules and sometimes blood cells in the endoplasm.
6. Active amoebae constantly change shape by thrusting out pseudopodia.
7. For differentiation of the most common amoebae see Table 25 and Figure 17.
8. The amoebae must be differentiated from the following:
   - a. Mononuclear phagocytic cells which sometimes contain erythrocytes. These phagocytic cells are not actively motile.
   - b. Epithelial cells.
9. Table 23 gives the differential types of stools found in dysenteries and ulcerative colitis.

Table 23. Types of Stools in Dysenteries and Ulcerative Colitis

	Amoebiasis	Bacillary Dysentery	Ulcerative Colitis
is	+++	+	++++
rocytes	+++	++++	++++
norphonuclears	+ (many pyknotic)	++++	++++
e mononuclear cells	+	+++ (may contain erythrocytes)	++
cot-Leyden crystals	+	0	0

Cystic Stage in Carriers.

When the trophozoites are extruded into the umen of the bowel, if the stool is formed or conditions are otherwise unfavorable for their continued existence in trophozoite form, they cease to ingest food, become spherical and enter a precystic stage. They then secret a cyst wall and slowly mature by nuclear division.

For differentiation of the various cysts see Table 25 and Fig. 17.

Cysts must not be confused with Blastocystis hominis which sometimes resembles cysts (see p. 128 and Fig. 17).

III. Flagellates.

Examination—Use same methods as for other Protozoa.

Giardia lamblia.

Trophozoite Form.

a. Pear-shaped disc with concavity (sucking disc) on one side of blunt end for attachment to epithelial cells.
b. The movement is darting with much lashing of the flagella, but the organism does not progress very far.
c. For further description see Table 24 and Fig. 17.
d. The trophozoites are rarely found in the stools, unless they are very liquid and still warm; they are occasionally found in aspirated duodenal contents.

Cyst Form.

a. The shrinking of the contents from the wall is a diagnostic feature.
b. The nuclei are in the anterior end.
c. For further description see Table 24 and Fig. 17.

Chilomastix mesnili.

Trophozoite Form.

a. Pear-shaped, rounded anteriorly, and pointed posteriorly.
b. Three flagella are attached to the anterior end and a fourth lies along the floor of the large mouth-like cavity or cytostome.
c. The trophozoite moves forward in a slow, steady manner, occasionally turning on its axis.
d. The large oval nucleus is near the anterior end.
e. A spiral groove runs obliquely across the ventral surface; this is characteristic of Chilomastix.
f. For further description see Table 24 and Fig. 17.

2. *Cyst Form.*
   a. Cyst wall is thickened at the narrow anterior end.
   b. For further description see Table 24 and Fig. 17.

D. Trichomonas hominis.

1. *Trophozoite Form.*
   a. Pear-shaped, rounded anteriorly, and pointed posteriorly, with a central axostyle which projects beyond the posterior end.
   b. The undulating membrane on one side terminates in a flagellum posteriorly.
   c. When in motion this membrane undulates and resembles a moving cog-wheel.
   d. For further description see Table 24 and Fig. 17.
2. *No cysts* are known.

E. Trichomonas vaginalis.

1. *Trophozoite Form.*
   a. Similar to T. hominis but larger.
   b. The undulating membrane extends only one half the length of the body where its marginal flagellum terminates.
   c. There is no free posterior flagellum, only the protruding axostyle.
   d. For further description see Table 24 and Fig. 17.
2. *No cysts* are known.

F. Enteromonas hominis.

1. *Trophozoite Form.*
   a. There are 3 anterior flagella and one posterior which adheres to the flattened side of the body for some distance and then projects free posteriorly or laterally.
   b. For further description see Table 24 and Fig. 17.
2. *Cyst Form.*
   a. Usually seen in the 2 nuclei stage.
   b. For description see Table 24.

TABLE 24. DIFFERENTIAL CHARACTERISTICS OF FLAGELLATES

	Giardia lamblia	Chilomastix mesnili	Trichomonas hominis	Trichomonas vaginalis	Enteromonas hominis	Embadomonas intestinalis
Trophozoite						
Shape	Pyriform.	Pyriform.	Pyriform.	Pyriform.	Ovoid.	Ovoid.
Length (microns)	9-21	6-24	8-15	8-30	4-10	4-9
Flagella	8 (4 anterior, 2 caudal, 2 ventral).	4 (3 anterior, 1 cytostomal).	5 (3 to 5 anterior, 1 posterior).	4 anterior	4 (3 anterior, 1 posterior).	2 anterior.
Undulating membrane	None.	None.	Present.	Present.	None.	None.
Axostyle	2	None (parastyle).	1	1	None.	None.
Spiral groove	None.	Present.	None.	None.	None.	None.
Cytostome	None.	Present.	Present.	None.	None.	Present.
Nucleus						
Number	2	1	1	1	1	1
Shape	Oval.	Spheroidal or oval.	Oval.	Oval.	Oval or cone-shaped.	Spheroidal or oval.
Karyosome	Large.	Small.	Large.	Not definite.	Large.	Medium.
Motion	Twisting—jerky.	Spiral—jerky.	Rotary—clockwise.	Rotary—clockwise.	Jerky.	Jerky.
Cyst						
Shape	Ellipsoidal.	Lemon-shaped or oval with knob at anterior end.	Unknown.	Unknown.	Elongate oval.	Pyriform.
Length (microns)	8-19	6-10			6-8	4-7
Nuclei	4-8	1			1-4	1
Flagella	4	1			None.	None.
Axostyle	2	None (parastyle).			None.	None.
Spiral groove	None.	Present.			None.	None.
Cytostome	None.	Present.			None.	Present.
Habitat	Duodenum, jejunum, and rarely gall-bladder.	Large and small intestine.	Large and small intestine.	Human vagina.	Large and small intestine.	Large and small intestine.

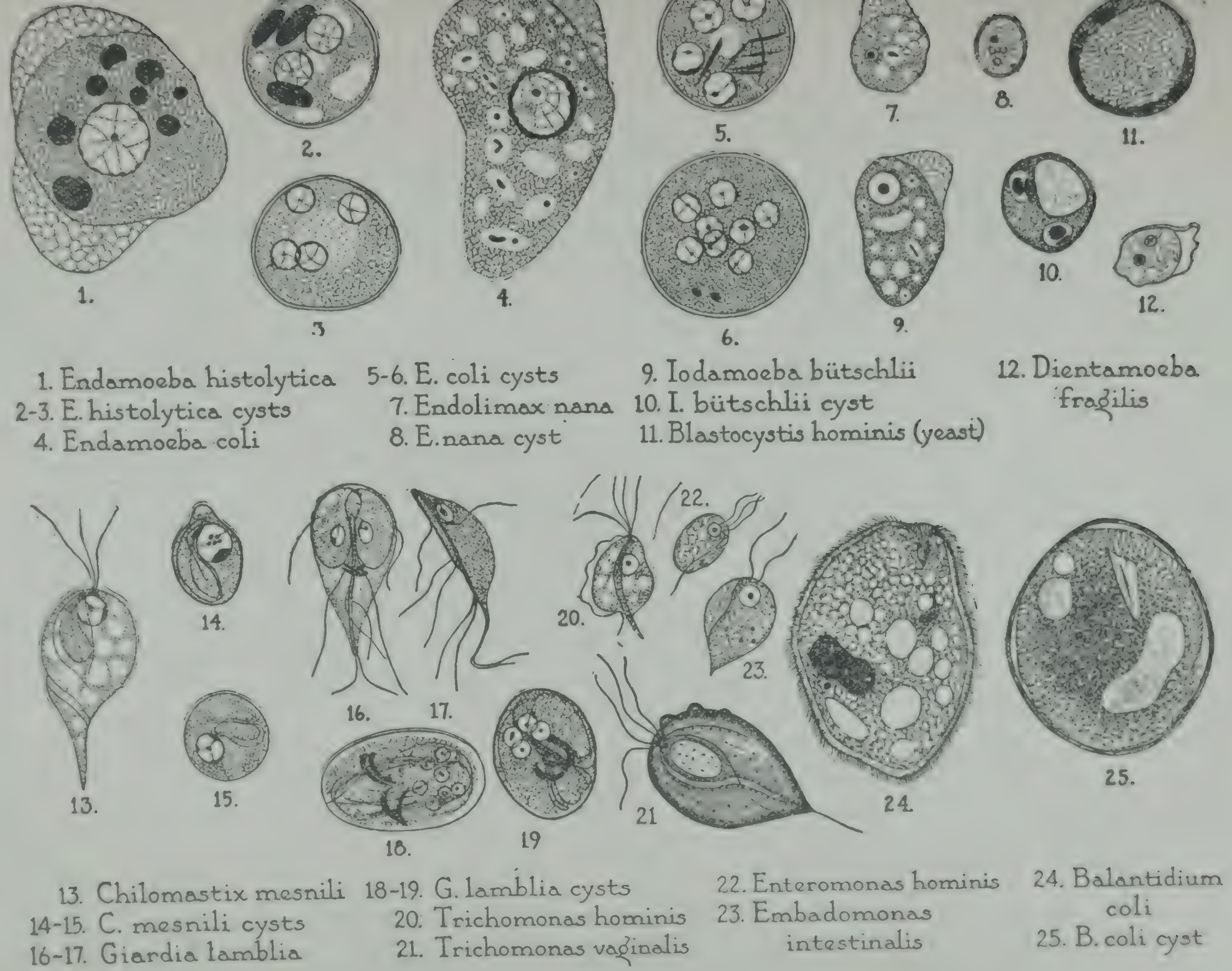

1.
2.
3
4.
5.
6.
7.
8.
9.
10.
11.
12.
1. Endamoeba histolytica
2-3. E. histolytica cysts
4. Endamoeba coli
5-6. E. coli cysts
7. Endolimax nana
8. E. nana cyst
9. Iodamoeba bütschlii
10. I. bütschlii cyst
11. Blastocystis hominis (yeast)
12. Dientamoeba fragilis
13.
14.
15.
16.
17.
18.
19
20.
21
22.
23.
24.
25.
13. Chilomastix mesnili
14-15. C. mesnili cysts
16-17. Giardia lamblia
18-19. G. lamblia cysts
20. Trichomonas hominis
21. Trichomonas vaginalis
22. Enteromonas hominis
23. Embadomonas intestinalis
24. Balantidium coli
25. B. coli cyst

Fig. 17. Protozoa.

(Reproduced from drawings by Dr. John F. Kessel in the *China Medical Journal* for February, 1925.)

TABLE 25. DIFFERENTIAL CHARACTERISTICS OF AMOEBA

VEGETATIVE OR TROPHOZOITE STAGE (Unstained)

	Endamoeba histolytica (Pathogenic)	Endamoeba coli (Nonpathogenic)	Endolimax nana (Nonpathogenic)	Iodamoeba butschlii (Nonpathogenic)	Dientamoeba fragilis (Nonpathogenic)
Size (microns)	20-35	20-40	6-10	9-14	8-10
Motion	Actively progressive and directional.	Sluggish, rarely progressive, not directional.	Sluggishly progressive in fresh feces but soon lost.	Sluggish, slightly progressive.	Slow indefinitely progressive or merely changing shape.
Pseudopodia	Bladelike, hyaline, explosive,ectoplastic. Nucleus moves out with pseudopodia.	Blunt, slowly formed, endoplastic, granular. Nucleus does not move out with the pseudopodia.	Blunt, formed slowly and many at same time. Hyaline and ectoplastic.	Blunt, formed slowly, ectoplastic and hyaline.	Large leaf-like, formed rapidly, ectoplastic, hyaline. Outline is often indistinct.
Ectoplasm	Hyaline, refractive, sharply differentiated from endoplasm.	Slightly refractive and poorly differentiated from endoplasm.	Not sharply differentiated from endoplasm.	Not sharply differentiated from endoplasm.	Clear.
Inclusion bodies	Erythrocytes present in endoplasm when stool contains blood. Few or no ingested bacteria, yeasts, or debris. Vacuoles scanty.	Endoplasm contains many bacteria and fecal particles but very rarely an erythrocyte. Numerous vacuoles.	Endoplasm contains bacteria but no erythrocytes. Numerous vacuoles.	Endoplasm contains bacteria and debris. Numerous vacuoles.	Endoplasm contains bacteria and debris. Numerous vacuoles.
Nucleus	Indistinct, often invisible. Thin-walled and evenly granular. Karyosome similar to one in cyst.	Distinct, thick-walled, not always spherical. Karyosome similar to one in cyst.	Indistinct, moderately thick-walled. Karyosome similar to one in cyst.	Sometimes distinct, thick-walled. Karyosome in center surrounded by refractive granules.	Usually 2 indistinct nuclei, tin walled. Karyosome large composed of 4 chromatin granules.

CYSTIC STAGE OF DEVELOPMENT

	Endamoeba histolytica	Endamoeba coli	Endolimax nana	Iodamoeba butschlii	Dientamoeba fragilis
Size (microns)	7-15	13-20	8-10	8-12	Cysts unknown.
Shape	Spherical or slightly oval, wall single and delicate in young cysts; thicker and sometimes double outlined in older ones.	Similar to E. histolytica, but double outline of wall more frequent and much more distinct. Entire cyst more refractive.	Oval, thin-walled.	Spheroidal or irregular, thick walled.	
Glycogen and chromatoid bodies	Cytoplasm of one and two-nucleated cysts granular, often with a poorly defined clear area which stains brown with iodine (glycogen). Presence of brightly refractive rod-shaped masses of chromatin is characteristic. Glycogen and chromatin disappear as cyst matures.	Similar, but glycogen more abundant and sharply defined when stained with iodine. Chromatin rarely present, like splinters of glass. Cytoplasm is evenly coarsely granular.	Glycogen sometimes present as a single mass, staining deeply with iodine. Chromatin absent.	Glycogen almost always present and large. Chromatin absent.	
Nuclei	Fully developed cysts usually contain 4 small nuclei seen by focusing at different levels. May have 1 or 2 nuclei. Minute karyosome in center.	Fully developed cysts usually contain 8 to 16 nuclei. May have 1, 2, or 4 nuclei. Large eccentric karyosome.	Fully developed cysts contain 4, rarely 8, very small nuclei with a large karyosome central or to one side.	One, rarely two, nuclei with refractive granules and karyosome at one end.	

hbadomonas intestinalis.

ophozoite Form.

The two anterior flagella arise from blepharoplasts on the nuclear membrane of the single nucleus which is near the blunt end of the parasite.

For further description see Table 24 and Fig. 17.

st Form.

There is a single nucleus and a shadowy outline of the cytostome.

For description see Table 24.

IV. Ciliates.

lantidium coli.

ophozoite Form.

Size—50 to 70 microns in length and 40 to 50 microns in breadth.

Ovoid shaped, narrower at anterior end where there is an invaginated cytostome or mouth; at the posterior end is an indistinct excretory opening or cytopyge.

The surface is covered by longitudinal rows of hair-like cilia, by means of which it moves forward with a rapid spiral movement.

d. The cytoplasm has a faint yellow or greenish color and contains many vacuoles.

e. The macronucleus is large and kidney-shaped, located near the center of the organism. The small micronucleus lies close to the concavity of the macronucleus.

2. *Cyst Form.*
   a. *Size*—45 to 65 microns in diameter.
   b. A thick double cyst wall encloses the resting organism similar to that of the motile form.
   c. See Fig. 17.
3. *Life Cycle.*
   a. Definitive host, hog; incidental host, man.
   b. Habitat of the adult balantidium is the mucosa of the large intestine.
      1) The organism reproduces by transverse binary fission.
      2) The micronucleus divides first, then the macronucleus, followed by division of the body.
   c. Cysts are formed and are excreted in the feces.
   d. The cysts are ingested by a new host.

B. There are no other important pathogenic ciliates.

Sputum

Sputum is the material coughed up from the bronchi and trachea and should not be contaminated with saliva or nasal secretions.

Macroscopic Examination

I. Collection of Sputum.

A. ***Collect*** an early morning specimen or the entire 24 hour specimen.

B. ***Instructions to Patient.***

1. The mouth should be rinsed well to avoid contamination with food particles.
2. The sputum must be coughed up from the lungs or bronchi and placed in a clean, wide-mouthed bottle (preferably sterile) with a screw top.
3. Particular caution must be taken to avoid smearing any of the sputum on the outside of the bottle.

II. Amount.

A. ***Normal***—none or too small to estimate.

B. ***Abnormal.***

1. Over 100 cc. in 24 hours may occur in pulmonary edema, lung abscess, bronchiectasis, advanced pulmonary tuberculosis, broncho-moniliasis, and pulmonary hemorrhage.
2. Over 500 cc. of an anchovy colored sputum in 24 hours suggests an amoebic abscess of the lungs or rupture of an amoebic abscess of the liver into the lungs.
3. Small amounts of sputum are obtained in diffuse bronchitis, early pulmonary tuberculosis, and some cases of lobar pneumonia.
4. An increase in quantity usually indicates progression of the disease, while a decrease may indicate healing. A sudden decrease may be due to plugging of the air passages.

III. Odor.

A. ***Normal*** sputum has no odor.

B. ***Abnormal.***

1. *Sweetish* in pulmonary tuberculosis with cavities, bronchiectasis, and broncho-moniliasis.
2. *Putrid* in gangrene of the lung, bronchiectasis, and lung abscess.
3. *Cheesy* in necrosis of malignant tumors and perforating empyemas.
4. Any sputum may become very foul after standing 12 hours or more.

IV. Appearance.

A. ***Color.***

1. *Gray*—due to pus, epithelial cells, etc.
2. *Yellow*—due to pus.
3. *Green*—due to bile pigment, a Ps. aeruginosa (B. pyocyaneus) infection, or rupture of a liver abscess into the lung.
4. *Red*—due to fresh blood from a hemorrhage.
5. *Rusty or brown*—due to old blood as in pneumonia, pulmonary gangrene, rupture of an amoebic abscess of the liver into the lung, or pigmented cells in chronic passive congestion.
6. *Black*—due to inhalation of dirt, coal dust, or to the decomposition of anthracotic tissue.

B. ***Consistency.***

1. *Serous*—colorless or yellow, generally frothy. Found in pulmonary edema.
2. *Mucoid*—glassy, transparent, and tenacious. Found in acute bronchitis, asthma, lobar pneumonia, and whooping cough.
3. *Purulent*—pus. Found in ruptured empyema, abscess, and some cases of bronchiectasis.
4. *Mucopurulent*—mucus and pus. Found in lung cavitation and in broncho-moniliasis.
5. *Tenacious*—thick and viscous due to mucus. Found in lobar pneumonia and in broncho-moniliasis.
6. *Bloody.*
   a. Sputum may be streaked with blood, contain pure blood, or have a rusty or prune-juice appearance (see color under A above).
   b. Hemoptysis (blood from the lungs) is bright red and frothy, while hematemesis (blood from stomach) is dark brown.
   c. Blood is found in sputum in mitral stenosis, pulmonary infarction, carcinoma of the lungs, pulmonary tuberculosis, bronchiectasis, and acute broncho-moniliasis.
   d. "Prune-juice" sputum is found in pneumonia.

ayer Formation.

If sputum is kept in a tall glass for several hours, some sputa separate into 2 or 3 distinct layers; a frothy mucus on top, a second layer of opaque watery material, and an underlying sediment of pus, tissue, bacteria, etc.

. Found in bronchiectasis, gangrene, and abscess of the lung.

V. Minute Macroscopic Examination.

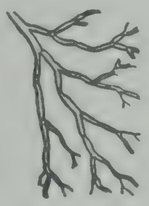
repare a portion of the sputum for examinaion by pouring it into a Petri dish to obtain thin layer.

xamine carefully against a black backround with the aid of a hand lens for the ollowing:

Cheesy masses are fragments of necrotic tissue. Found in tuberculosis, pulmonary gangrene, abscess, or actinomycosis.

Curschmann's spirals—see microscopic examination.

Bronchial casts are white branching tree-like casts composed of fibrin.



a. The size varies according to the bronchi in which they are formed.
b. They are usually rolled up into a ball and can be seen best when floating on water.
c. Found in fibrinous bronchitis and consolidation of pneumonia.

Dittrich's plugs are yellowish white plugs from bronchi or bronchioles.

a. They vary in size from that of a pin-head to a bean and have a very putrid odor.
b. They consist of cellular debris, fat globules, fatty acid crystals, and bacteria.
c. Found in bronchial asthma, putrid bronchitis, and bronchiectasis.

Lung stones or broncholiths are calcified stagnant contents of cavities or dilated bronchi or are calcified tuberculous material.

Select any of the above, if present, for microscopic examination.

Microscopic Examination

I. Unstained Sputum.

Select a portion of the sputum during the minute macroscopic examination described above and place on a slide, apply a cover glass, and examine with low and high dry objectives.

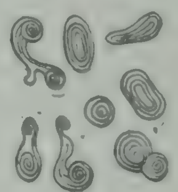
Pus and blood may be distinguished as readily in unstained films of sputum as in a urine sediment.

C. *Pigmented Cells.*

1. *Heart-failure Cells.*
   a. Large mononuclear cells containing hemosiderin, a yellow to brown granular pigment which gives the Prussian blue reaction.
      1) Add a drop of 10% potassium ferrocyanide to a wet or dry preparation, let stand for a few minutes, and then cover with a drop of 5% HCl.
      2) The hemosiderin granules become blue, but many of the granules will fail to take the blue color.
   b. Found in passive pulmonary congestion due to mitral stenosis, cardiac decompensation, also in pulmonary infarction, or after pulmonary hemorrhage.

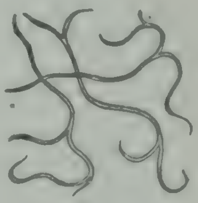
2. *Carbon-laden Cells.*
   a. Cells containing black granules.
   b. Found in anthracosis, and in morning sputum of those who inhale large amounts of tobacco smoke or who live in a smoky atmosphere.

D. *Myelin Globules.*



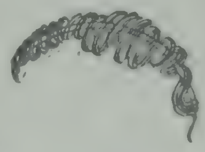
1. They are colorless, highly refractive, round, oval, or pear-shaped globules of various sizes, often resembling fat droplets; some large ones show concentric or irregularly spiral markings.
2. They are found both free and within cells; the intracellular globules are small giving the cells the same appearance as heart-failure cells.
3. They have no clinical significance but may be confused with blastomyces.

E. *Elastic Fibers.*



1. These appear as faint yellow, slender, highly refractive, double contoured, wavy fibers with curved and split ends.
2. They indicate breaking down of the lung parenchyma.

F. *Curschmann's Spirals.*



1. The spirals are yellow wavy threads, usually coiled into balls.
2. They are from 1.5 to 5 cm. in length.
3. Under low power they appear as mucous threads with a bright colorless central line about which are wound many fibrils.
4. The fibrils are sometimes loosely, sometimes tightly wound and may contain eosinophils and Charcot-Leyden crystals.
5. They suggest bronchial asthma.

G. *Crystals.*

1. Illustrations of cholesterol, leucine, and tyrosine crystals may be found in the Section on Urinalysis, page 21, and Charcot-Leyden, fatty acid and hematoidin crystals may be found in the Section on Feces, page 127.
2. *Charcot-Leyden*—are colorless, hexagonal, sharply pointed, often needle-like crystals. They suggest bronchial asthma.
3. *Fatty acid*—are short slender needles generally massed, so that the outlines of the separate crystals are obscure. Found in gangrene, putrid bronchitis, and chronic tuberculosis.
4. *Cholesterol*—are large flat plates with one or more corners cut out. Found in empyema, chronic lung abscess, and chronic tuberculosis.
5. *Leucine* — are highly refractive, yellow spheres resembling fat globules. Found after rupture of an empyema into the lung.
6. *Tyrosine*—are colorless, fine, radiating needles, the masses being dark in the center. Found with leucine crystals.
7. *Hematoidin*—are yellowish or brown crystals, needle-like or rhombic in shape. Found after hemorrhage in the lung.

H. *Fungi.*

1. See Section on Mycology, page 204.
2. *Actinomyces bovis*, see Fig. 23A, page 210.
    a. Small, gray or yellowish, "sulfur" granules, 1 mm. or less in diameter, can be detected by direct examination with a hand lens.
    b. Crush one of these granules on a slide and add a drop of 20% NaOH.
    c. Examine with a low power objective for a circular mass made up of filaments which radiate from a compact center.
    d. The filaments end in "clubs" which are arranged around the periphery of the mass.
3. *Nocardia asteroides and gypsoides* occur as short, slender branching filaments which are usually in small tangled clusters. The filaments do not have clubs and are moderately acid-fast, therefore may be mistaken for tubercle bacilli.
4. *Blastomyces dermatitidis* occurs as round or oval yeast-like bodies with a double contoured, highly refractive capsule, often budding, see Fig. 23C and D, page 210.
5. *Leptotrichia buccalis* occurs as long unbranched filaments with partitioned walls.
6. *Aspergillus fumigatus* consists of a network of broad mycelial threads some of which enlarge at the tip forming a vesicle. Flask-shaped sterigmata are produced from the vesicle which in turn produce unbranched chains of conidia, see Fig. 22R, page 205
7. *Penicillium* consists of a network of mycelial threads some of which form conidiophores. The conidiophores branch to give a brush-like appearance; these branches produce flask-shaped sterigmata from which unbranched chains of conidia are formed, see Fig. 22S, page 205.
8. *Monilia (Candida)*, see Fig. 24C and D, page 214.
    a. Appear as yeast-like budding cells.
    b. A tangled network of fine mycelial threads with clusters of spores may be present.
    c. C. albicans is the only one considered pathogenic for man.
9. *Coccidioides immitis* is similar to blastomyces but does not bud. The organism contains many small spores in the center, see Fig. 23E, page 210.
10. *Cryptococcus neoformans* (Torula histolytica) occurs as an ovoid to spherical, single-budding, thick-walled, yeast-like organism, 5 to 20 microns in diameter and surrounded by a wide, refractive gelatinous capsule, see Fig. 23F, page 210.
11. *Geotrichum* appears as oblong or rectangular cells, 4 by 8 microns, with somewhat rounded ends or as large spherical cells, 4 to 10 microns in diameter.

I. *Parasites.*

1. Endamoeba histolytica are found rarely in amoebic abscess of the lung or after the rupture of a liver abscess into the lung. (See Table 25 and Fig. 17.)
2. Scolices and hooklets of Echinococcus granulosus may be found after rupture of a hydatid cyst of the lung or the rupture of a cyst in the liver into the lung. (See Table 21 and Fig. 15.)
3. Larvae of Strongyloides stercoralis, hookworm, and roundworm may be found. (See Table 20 and Fig. 14.)
4. Ova of Paragonimus westermani (lung fluke) may be found. (See Table 22 and Fig. 16.)
5. Rarely Trichomonas hominis is found in sputum from putrid bronchitis and gangrene of the lung, see Fig. 17.

II. Stained Sputum.

A. *Tubercle Bacillus* (Ziehl-Neelsen Stain).

1. Select a small cheesy and purulent mass from the sputum and make a thin smear, dry, and fix in a flame. Do not overheat.
2. Apply as much carbol-fuchsin solution as will stay on the slide, and steam (do not boil

er a flame for 5 minutes, replacing stain it evaporates. (Do not let stain evaporate mpletely.)

t the stain cool until a sheen appears on e stain.

ash with water and decolorize with acid cohol (3% HCl in 95% alcohol) until all e pink has disappeared from the thinner rtions of the smear.

ash and counterstain with Loeffler's meth- ene blue solution for 1 minute.

ubercle bacilli are acid-fast and retain the d stain.

they cannot be found in such a smear, the utum should be concentrated by one of e digestion methods.

smear should be examined thoroughly be- ore calling it negative. At least 3 sputa ob- ined on different days should be examined efore the case is considered negative.

ecord a positive slide as follows:

+ = from 3 to 9 acid-fast bacilli in entire smear.
+ + = 10 or more bacilli in entire smear.
+ + = average of 1 to 10 bacilli in each field.
+ + = average of more than 10 bacilli per field.

lways note on the report whether the smear as made directly from the sputum or after oncentration of the sputum.

am Stain.

ake a smear, dry, and fix in a flame.

ake a Gram stain (see Section on Bacteri- gy, p. 164).

port morphology and staining reaction of ganisms found.

right's Stain.

ake a thin smear and stain like a blood smear cept increase the staining time.

amine for the following:

Polymorphonuclear neutrophils are the predominating cell in pyogenic infections.

Eosinophils or eosinophilic granules are found in bronchial asthma.

Lymphocytes predominate in early or mild cases of tuberculosis.

Erythrocytes are only present after a hemorrhage or in inflammatory conditions.

Epithelial cells.

1) *Squamous cells* are large flat polygonal cells with a small nucleus; they are frequently studded with bacteria. They come from the mouth and upper air passages.
2) *Cylindrical cells* come from the nose, trachea and bronchi. Occasionally ciliated cells from the trachea and bronchi are seen.
3) *Septal cells* are large round or oval cells, 20 to 40 microns, with one or two rounded nuclei.

Digestion Methods

I. Sodium Hydroxide Method.

A. *Method.*

1. Incubate the sputum at 37°C. for 3 hours or overnight. (This step aids in the digestion but may be omitted if time does not permit.)
2. Pour the sputum into a 50 cc. chemically clean centrifuge tube and add approximately an equal amount of digesting fluid (2% NaOH containing 0.2% potassium alum and 0.002% bromthymol blue).
3. Mix thoroughly with a wooden applicator and incubate at 37°C. for 30 minutes, stirring occasionally to insure complete liquefaction.
4. Add slowly approximately 1 N HCl (10% by volume) with constant stirring until a greenish yellow color appears. It will take approximately one half of the volume of NaOH used.
5. Centrifuge at high speed for 45 minutes.
6. Pour off the supernatant fluid, make a smear of the sediment, and stain for tubercle bacilli.

B. *Cultures and Guinea Pig Inoculation.*

1. Make cultures from the sediment.
2. Dilute the remaining sediment with 2 cc. of sterile 0.85% NaCl solution and inject subcutaneously into the groin of a guinea pig.

II. Oxalic Acid Method.

A. *Method.*

1. Mix thoroughly equal parts of sputum and 5% pure oxalic acid solution.
2. Incubate at 37°C. for 30 minutes, stirring occasionally during this time.
3. Dilute with 4 volumes of sterile 0.85% NaCl solution and mix thoroughly.
4. Centrifuge at high speed for 45 minutes.
5. Decant the supernatant fluid, make a smear of the sediment, and stain for tubercle bacilli.

B. *Cultures and Guinea Pig Inoculation.*

1. Add sterile 0.85% NaCl solution to the sediment, shake, and centrifuge again at a high rate of speed for 45 minutes.
2. Pour off all the supernatant fluid, make cultures, then dilute the sediment with 2 cc. of sterile NaCl solution, and inject subcutaneously into the groin of a guinea pig.

Bacteriological Examination

I. Cultures—see page 175.

II. Pneumococcus Typing—see page 190.

III. Autogenous Vaccine—see page 199.

IV. Guinea Pig Inoculation—see page 200.

Cerebrospinal Fluid

General Considerations

I. Normal Cerebrospinal Fluid.

A. ***The chief source of the fluid*** is the highly vascular choroid plexus in the ventricles of the brain.

1. A large portion is produced by filtration from the blood plasma through a selectively permeable membrane consisting of the lining of the plexus.
2. It is thought that a small portion is secreted by the cells of the choroid plexus and by the ependymal cells lining the ventricles.

B. ***Location of the Fluid.***

1. Internally it fills the ventricles of the brain (ventricular fluid), the cisternae (cisternal fluid), and the canal of the spinal cord.
2. Externally it fills the space between the pia and arachnoid membranes surrounding the brain and spinal cord (spinal fluid).

C. ***Function of the Fluid.***

1. It acts as a medium for the transfer of substances from the tissues of the brain and spinal cord to the blood stream.
2. It serves as a space compensating mechanism in regulating the contents of the cranium.
3. It is a fluid buffer against injury.

II. Amount and Pressure.

A. ***Amount.***

1. The normal amount is roughly estimated to be about 1 cc. per pound of body weight.
2. There is an increased amount in acute and chronic congestion of the meninges due to increased transudation of plasma through the capillaries and probably to increased permeability of the choroid plexus.
3. There is also an increased amount in acute and chronic infections due to the production of inflammatory exudate and to increased permeability of the capillaries.

B. ***Pressure.***

1. Normal pressure for the horizontal position varies between 70 and 200 mm. of water (0-8 mm. of mercury) with an average of 100 to 150 mm.
2. For abnormal findings see Table 26, page 154.

III. Collection of Fluid.

A. ***Lumbar puncture*** is a relatively safe and simple procedure, but should not be done unless there are definite indications.

1. *Indications.*
   a. *Diagnostic.*
      1) To obtain spinal fluid for study.
      2) To estimate intracranial pressure.
      3) To test for spinal block.
      4) To introduce air or a lipoidal substance.
   b. *Therapeutic.*
      1) To introduce serum, penicillin, streptomycin, or an anesthetic.
      2) To remove blood or irritative exudates.
2. *Contraindications.*
   a. Subtentorial tumors.
   b. Presence of greatly increased intracranial pressure.

B. ***Cisternal puncture* (cisterna magna)** is somewhat more dangerous than a lumbar puncture and is usually done only under the following conditions:

1. Block in the spinal canal.
2. Deformity of the vertebrae.
3. Infection of the tissues of the back.

C. ***Ventricular puncture*** is frequently done in infants who have open fontanelles but rarely done in adults except in connection with ventriculography.

D. ***Amount to be Collected.***

1. At least 8 to 10 cc. of fluid is necessary for a complete examination.
2. It should be collected in 3 sterile, chemically clean test tubes, numbered 1, 2, and 3.
3. The first drops are placed in tube 1 and may contain some blood from the puncture.
   a. This fluid should not be used, unless it is necessary for bacteriological examination.
   b. The presence of the slightest amount of blood makes the various laboratory tests valueless, because the cell count and chemical composition (except chlorides) of spinal fluid are normally lower than those of blood.
4. Collect 7 cc. in tube 2 for serological, bacteriological, and chemical tests.

llect about 2 cc. in tube 3 for cell count l qualitative protein tests.

hen a xanthochromic (canary yellow) fluid obtained, it is advisable to add a trace of nium oxalate to tubes 2 and 3 to prevent otting.

ne cell count and examinations for bac- ia and sugar must be done at once, while e remaining tests can be delayed several urs if the specimen is kept in the refriger- or.

enous blood should be drawn at the same ne as the spinal fluid if chemical tests are to done, especially for sugar and chloride.

Macroscopic Examination

I. Physical Properties.

olor.

ormally the fluid is as clear and colorless distilled water.

ight red due to fresh blood from a vessel nctured while inserting the needle; upon ntrifugation the supernatant fluid is clear.

ull red or brown (depending on the age of e lesion) in hemorrhages due to a frac- red skull or in some intracranial hemor- ages and chronic hemorrhagic pachymenin- tis. After 48 hours hemolysis begins, so at a hemorrhage of at least this age is rec- gnized by the yellow or red color of the pernatant fluid after centrifugation.

ellow (xanthochromic) may be due to ood pigments resulting from disintegration erythrocytes within the subarachnoid space to altered permeability of the lining mem- ranes to pigments in the blood plasma which nder normal conditions are excluded.

Found in subarachnoid hemorrhage, extradural and subpial hematoma, spinal block, tumor, abscess, acute inflammation, carotenemia, and acute toxoplasmosis.

In severe and chronic jaundice the fluid may contain bile.

reenish or grayish due to pus cells in severe flammatory reactions.

urbidity.

acute meningitis the fluid may exhibit arying degrees of cloudiness, from slight rbidity to the opacity of almost pure pus. the less acute stage of epidemic menin- tis, it is sometimes quite clear.

is usually clear in tuberculous and syph- tic meningitis, tabes, and poliomyelitis.

eaction is alkaline.

pecific Gravity: 1.003-1.008.

II. Coagulation.

A. ***Normal*** spinal fluid does not coagulate.

B. ***Abnormal.***

1. The fluid clots when there is an increase in proteins including fibrinogen.
2. Numerous small coagula occur in paresis.
3. A "cobweb" or "pine tree" delicate coagulum is typical of tuberculous meningitis; it forms on the surface of the fluid and extends down the middle of the tube.
   a. Twelve or more hours may be required for its formation.
   b. The absence of a pellicle does not exclude tuberculous meningitis.
4. Heavy coagulum and sediment occur in acute suppurative meningitis.
5. Complete coagulation with xanthochromia without hemorrhage occurs in Froin's syndrome (spinal subarachnoid block).

Chemical Examination

I. Proteins (Qualitative Tests).

A. ***General Considerations.***

1. The protein of chief interest is globulin.
2. A test for globulin is valueless when applied to fluid containing blood, owing to the presence of serum globulin.
3. If the fluid is cloudy, it should be centrifuged and the clear supernatant fluid used for the test.

B. ***Nonne-Apelt Test.***

1. *Globulin* (Same as Ross-Jones Test).
   a. Pipette 1 cc. of spinal fluid into a small test tube.
   b. Place the tip of a pipette containing 1 cc. of a saturated solution of ammonium sulfate (80 gm. in 100 cc. of distilled water) to the bottom of the test tube, holding the finger over the top of the pipette.
   c. Gently remove the finger so that the ammonium sulfate solution layers underneath the spinal fluid and then place the finger over the top of the pipette and remove the pipette from the fluid.
   d. If positive, a clear-cut, thin, grayish-white ring appears at the zone of contact of the two fluids within a few seconds.
   e. Observe for 3 minutes. If a ring is formed at the zone of contact, shake the tube to mix the fluid with the ammonium sulfate and report as follows:

+ = a ring appearing within 3 minutes which is visible only against a dark background and leaving no trace on mixing the contents of the tube.
++ = a faint opalescence after mixing.
+++ = a definite cloud after mixing.
++++ = a heavy cloud after mixing.

f. Normally a ring may appear after 3 minutes.

2. *Albumin.*
   a. Shake the contents of the tube used in the globulin test and filter.
   b. Acidify with 1 drop of 10% acetic acid and boil.
   c. A slight cloudiness is normal.
   d. Report as follows:

Neg. = a slight cloudiness.
+ = a definite cloudiness with a fine precipitate.
++ = a flocculate precipitate in a slightly cloudy fluid.
+++ = a heavy flocculate precipitate in a clear fluid.

C. *Pandy's Test.*

1. To 1 cc. of a saturated aqueous solution of phenol, add 1 large drop of spinal fluid.
2. A bluish-white cloud forming immediately around the drop indicates an abnormal amount of globulin. Normal fluids may show a faint trace but this should be reported negative.
3. A saturated solution of phenol is prepared by placing 10 cc. of melted phenol in a bottle and adding 90 cc. of distilled water. Shake at intervals for several days during which time it is kept in the incubator at 37°C.

II. Total Protein (Quantitative Test).

See Section on Chemistry, page 277, for method and Table 26 for values and significance.

III. Sugar.

A. *General Considerations.*

1. The test for sugar must be performed within one-half hour after withdrawal of the fluid, because glucose gradually decomposes on standing.
2. Blood for glucose determination must be drawn at the same time.
3. If possible, the spinal fluid and blood for this test should be drawn before breakfast.
4. The glucose in spinal fluid is normally 60% of that in the blood.

B. *Method*—See Section on Chemistry, page 269.

C. *Interpretation*—See Table 26 on page 154.

IV. Chlorides.

A. *General Considerations.*

1. Blood for chloride determination should be drawn at the same time the spinal fluid is withdrawn.
2. The chlorides in spinal fluid is normally 25% higher than that in the blood.

B. *Method*—See Section on Chemistry, page 298.

C. *Interpretation*—See Table 26, page 154.

Cytology

The leukocyte count in the spinal fluid must be made within one hour after the spinal puncture, because cells disintegrate on standing. The routine examination should include both a total and a differential count.

I. Cell Count.

A. Shake the spinal fluid well and place about 0.4 cc. in a small test tube or on a watch glass so that the remaining fluid will not be contaminated with the diluting fluid.

B. *Method I.*

1. Draw up the diluting fluid in the leukocyte pipette to the mark 1 and fill to mark 11 with spinal fluid.

Diluting Fluid:

Crystal violet	0.1 gm.
Glacial acetic acid	10.0 cc.
Distilled water	90.0 cc.

Filter; the diluting fluid should be free of any particles.

2. Mix by shaking the pipette well and then discard 2 or 3 drops.
3. Place a drop on each side of a double counting chamber in the same manner as for a leukocyte count and wait 2 minutes for the cells to settle.
4. Count the cells in the 18 one-millimeter squares (9 on each ruling) and multiply by 0.6. The result will be the number of cells per c. mm. of undiluted fluid.
5. Differentiate the cells while counting if the count is above normal. Report per cent of each type.
6. The normal count varies from 1 to 8.
7. An increased cell count is due to an irritative or inflammatory lesion of the brain, spinal cord, or meninges.
8. A Fuchs-Rosenthal counting chamber may be used instead of a hemocytometer counting chamber.
   a. The cells must settle 5 minutes before counting.
   b. The ruled area covers 16 sq. mm. and with the cover glass on it has a depth of 0.2 mm.
   c. The number of cells counted in the 16 sq. mm. is multiplied by 0.35 to obtain the number per c. mm.

Method II (Pollock's Method).

Draw glacial acetic acid up in the erythrocyte pipette to mark 1, suck up into bulb, hen draw more acid to mark 1.

ill pipette with spinal fluid, shake well, and iscard 5 or 6 drops.

lace a drop on each side of a double count- ng chamber.

Count all cells present in 18 one-millimeter quares (9 on each ruling) distinguishing be- ween erythrocytes and leukocytes. The nu- lei of the leukocytes will be distinct, while he erythrocytes will have faint outlines. Multiply the result by 0.57 to obtain the number of cells per c. mm.

A correction can be made when the spinal luid is bloody by making an erythrocyte and eukocyte count on the patient's blood.

Multiply the ratio of the erythrocyte count of the spinal fluid to the erythrocyte count of the blood by the blood leukocyte count and subtract this product from the leukocyte ount of the spinal fluid.

$$\text{W.B.C. (sp. fl.)} - \frac{\text{R.B.C. (sp. fl.)} \times \text{W.B.C. (blood)}}{\text{R.B.C. (blood)}}$$

=W.B.C. per c. mm. of spinal fluid.

II. Differential Count.

Method.

Centrifuge the fluid for 10 to 15 minutes at moderate speed.

Pour off the supernatant fluid into another est tube and make thin smears of the sediment on a slide.

Dry in the air without heat and make a Wright's stain.

Count and tabulate 100 leukocytes.

f the fluid was xanthochromic and obtained rom an infant less than 2 months old, examine particularly for toxoplasma organisms in the olymorphonuclear leukocytes or monocytes, ee page 79.

Normally, only mononuclear cells (lymphocytes or an occasional monocyte) and an ccasional endothelial cell from the lining of he pia-arachnoidal spaces are found.

Bacteriology and Serology

I. Smears.

Direct.

Centrifuge the fluid at a high rate of speed or 15 minutes.

Make a smear, dry, fix with heat, and stain y Gram's method.

***mear for Tubercle Bacilli* (Cone's Meth- d).**

1. Place a paper clip in a small staining dish (about 4.5 cm. in diameter and 2 cm. deep) and place a No. 1 cover glass (22 x 30 mm.) on top of it.
2. Fill the dish with spinal fluid (10-15 cc.) and allow to stand at room temperature for several hours or until a fine web forms on the surface of the fluid.
3. Remove the fluid from the dish by means of a capillary pipette with the tip to the bottom of the dish.
4. As the fluid is removed the web will settle on the cover glass. (If no web forms, centrifuge the fluid for 45 minutes and make a smear of the sediment.)
5. Dry, fix by heat, and stain for 5 minutes by placing in a staining dish containing hot carbol-fuchsin solution.
6. Remove cover glass, rinse with water, and decolorize by repeatedly flooding the cover glass with acid alcohol until no more pink remains on the cover glass.
7. Rinse cover glass in water and counterstain with methylene blue solution for 1 minute.
8. Wash in water and dry.
9. Mount the cover glass with the smear side down on a glass slide with clarite.

II. Cultures and Guinea Pig Inoculation.

A. *Cultures.*

1. See Section on Bacteriology, page 183.
2. Cultures must be made immediately after the fluid is withdrawn; if this is impossible, the fluid must be kept in the 37°C. incubator until cultures can be made.

B. *Guinea Pig Inoculation.*

1. Centrifuge at a high rate of speed for 45 minutes and pour off all but 2 cc. of the supernatant fluid.
2. Shake to make a suspension of the sediment and inoculate into the groin of a guinea pig; 1 cc. subcutaneously and 1 cc. intraperitoneally.
3. See guinea pig inoculation in Section on Bac teriology for positive autopsy findings.

III. Wassermann.

See Section on Serology, pages 228 and 233.

Lange's Colloidal Gold Test

I. Colloidal Gold Test.

A. *Principle:* The changes in color of the colloidal gold are the result of differences in the aggregation of colloidal particles due to varying quantities of gamma globulin in the spinal fluid.

TABLE 26. CEREBROSPINAL FLUID FINDINGS IN DISEASE

Spinal Fluid

Disease	Pressure (mm. of water)	Appearance	Cells	Protein		Sugar (mg. per 100 cc.)	Chlorides (mg. per 100 cc.)	Lange	Remarks
				Qualitative (globulin)	Quantitative (mg. per 100 cc.)				
Normal	Patient in horizontal position 70-200 (average 100-150).	Clear. Colorless. No clot.	0-8 mononuclears.	0	25-45 (A/G ratio 5-1).	40-70 (60% of blood sugar).	720-750 (25% higher than blood chlorides).	Neg.	Urea nitrogen—6-15 mg. per 100 cc.
Epidemic Meningitis	200-700	Milky opalescent to yellow green. Thick clot.	1000-2000 or more. 95% polys in early stage.	++to+++	100-400	0-15	*Early* 650-860 *Late* 640-720	Meningitic curve.	Increased fluid. Very few meningococci found on smear. Found in cultures.
Pneumococcus Meningitis	Greatly increased.	Turbid to yellow. Clots.	*Acute cases* sl. increase. *Less acute cases* 100-5000, 95% polys.	++to+++	100-200 or higher.	0-10	600-650	Meningitic curve.	Pneumococci found on smear and culture.
Influenzal Meningitis	Greatly increased.	Sl. cloudy to turbid. Clots.	200-3000 60-70% polys.	++to++++	Markedly increased.	Diminished but not absent.	640-720	Meningitic curve.	H. influenzae hard to find on smear or culture.
Pyogenic Meningitis	Greatly increased.	Turbid. Thick clot.	100-6000 95% polys.	++to++++	Markedly increased.	0-15	630-680	Meningitic curve.	Strep., staph., typhoid, or coli may be found on smear or culture.
Tuberculous Meningitis	500-1000	Clear, opalescent or white. Fibrin web.	*Children* Early 10-100 Late 100-1000 70-90% mononuclears. *Adults* fewer cells.	±to+++	30-400 Highest readings shortly before death.	15-20	*Early* 680-700 *Late* 500-650	Meningitic curve.	Increased fluid. Tubercle bacilli may be found in web.
Lymphocytic Choriomeningitis	Increased.	Clear or opaque.	*Early* 10-100 *Late* 100-500 lymphocytes.	+	45-60	Normal.	Normal.	Meningitic curve.	Etiologic agent a virus.
Acute Poliomyelitis	Usually increased.	Clear to milky. Occ. a fibrin clot.	*Pre-paralytic* 15-2000 polys. *Paralytic* 10-100	±to++	*Pre-paralytic* 25-60 *Paralytic* 60-300	Normal.	Normal.	Variable.	After 3rd week cells less than 10. Etiologic agent a virus.

Encephalitis	or more.	fibrin clot.	monocytes. Less than 10 in 30 to 50% of cases.			normal 65-120	increased.	meningitic curve.	a virus.
Acute Syphilitic Meningitis	Increased.	Clear to turbid. Fibrin clot.	10-500 90% mononuclears.	±	25-60	Normal or sl. increase.	650-720	Tabetic or meningitic curve.	Blood Wass. pos. Sp. fluid Wass. neg.
Chronic Syphilitic (Vascular) Meningitis	Normal or sl. increased.	Clear.	10-60 mononuclears.	+	45-100	Normal.	Normal.	Tabetic curve.	Sp. fluid Wass.— 10 to 15% positive.
Syphilis (Tabes dorsalis)	Normal or slightly increased.	Normal.	10-75 mononuclears.	± to +	25-45	Normal or reduced.	Normal.	Tabetic curve.	Sp. fluid Wass.— 60 to 70% positive with 1 cc. of fluid.
Syphilis (Paresis)	Normal or slightly increased.	Normal. Usually small clot.	20-400 mononuclears.	+ to ++	50-100	Normal or reduced.	Normal.	Paretic curve.	Sp. fluid Wass.— 100% positive in all dilutions.
Brain Abscess	Usually increased.	Normal or slightly turbid.	*Ruptured* 10-100 70-75% polys. *Unruptured* 5-30 90-95% mononuclears.	± to +	30-100	Normal or increased.	*Ruptured* decreased. *Unruptured* normal.	Variable.	Organisms not usually found.
Brain Tumor	Usually increased.	Normal or xanthochromic.	Normal or 10-80.	± to ++	50-200	40-100	Normal.	Variable.	Normal fluid in about 20% of cases.
Subarachnoid Hemorrhage or Cerebral Injury.	Slightly increased.	Bloody or xanthochromic.	Cells increased due to blood.	+ to ++	45-200 or higher.	Normal or increased.	Normal or sl. decreased.	Variable	Chemical changes depend on amount of blood present.
Spinal Cord Compression	Normal or decreased.	Clear or xanthochromic.	Normal or sl. increase in mononuclears.	+ to ++++	*Complete block* 300-2000 *Partial block* 45-300	Normal.	Normal.	Variable.	*Complete block* Queckenstedt test—positive. *Partial block* Queckenstedt test—slow fall in pressure.
Multiple Sclerosis	Normal or decreased.	Normal.	70-90% of cases are normal. Others 5-50 mononuclears.	0 to +	30-80 (10-40% of cases are above normal).	Normal.	Normal.	50-65% paretic curve. 10-30% tabetic curve.	Sp. fluid normal in about 50% of all cases.

Meningismus is a clinical entity—spinal fluid is normal.

Table 26. Continued

	Appearance	Cells	Protein		Sugar (mg. per 100 cc.)	Chlorides (mg. per 100 cc.)	Lange	Remarks
			Qualitative (globulin)	Quantitative (mg. per 100 cc.)				
				Ventricular Fluid				
Normal	Clear.	0-4 mononuclears.	0	10-30	60-90	720-750	Neg.	Protein is increased on the side of a tumor and in obstructive hydrocephalus and decreased in nonobstructive hydrocephalus.
				Cisternal Fluid				
Normal	Clear.	0-8 mononuclears.	0	20-40	50-80	720-750	Neg.	Rarely obtained for diagnostic purposes.

B. *General Considerations.*

1. Spinal fluid containing blood cannot be used, because falsely positive and variable reactions may be obtained.
2. If the spinal fluid cell count is above normal, the fluid should be centrifuged and the supernatant fluid used for the test.

C. *Method.*

1. Arrange a series of 12 chemically clean, dry, pyrex test tubes in a rack with an opaque glass back.
2. Place 0.9 cc. of freshly prepared 0.4% NaCl solution in the first test tube and 0.5 cc. in each of the next 10 tubes. (Prepare the 0.4% NaCl solution by adding 1 cc. of a stock 10% NaCl solution to 24 cc. of distilled water.)
3. Place 0.85 cc. of a 1% NaCl solution in the 12th tube.
4. To the first tube add 0.1 cc. of the spinal fluid, which must be free from any trace of blood.
    a. Mix well by sucking the fluid up into the pipette and expelling it 4 times, and transfer 0.5 cc. to the second tube.
    b. Mix in a similar manner and transfer 0.5 cc. to the third tube; repeat this in each successive tube to and including the tenth.
    c. Discard 0.5 cc. from the tenth tube.

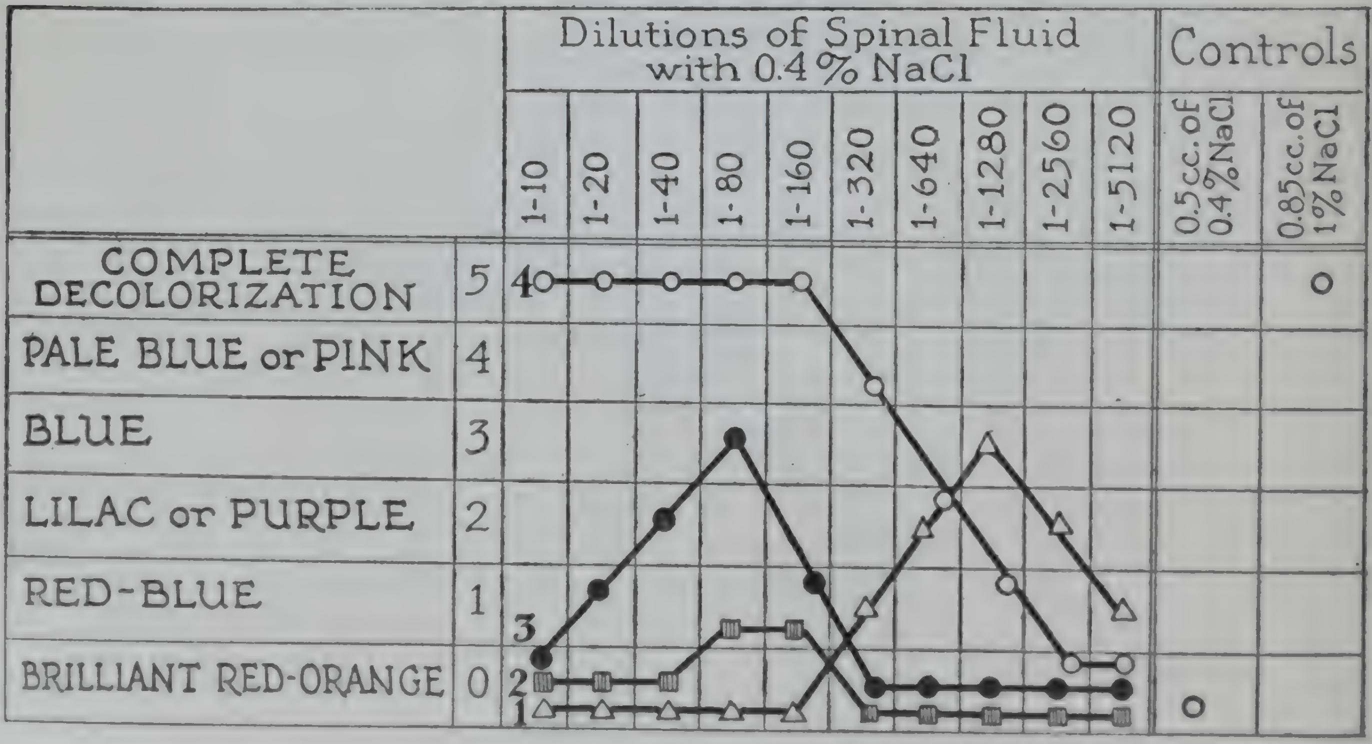


Fig. 18. Typical curves in Lange's colloidal gold test. 1. Meningitic. 2. Normal. 3. Luetic or tabetic. 4. Paretic

The 11th and 12th tubes are controls and contain no spinal fluid.

o each of the 12 tubes, add 2.5 cc. of the olloidal gold solution; the 11th tube serves s a control for the stability of the colloidal old, while the 12th is a control for the ensitivity of the colloidal gold. (The 12th ube should become colorless in 1 hour's ime.)

et stand overnight and read the next morn-ng.

The reaction in each tube is reported in the rder in which the tubes stand, from 1 to 10. They may be charted as in Fig. 18 or reported umerically as follows: normal, 0001100000; aretic curve, 5555542100; luetic or tabetic urve, 0123100000; meningitic curve, 0000012321; curve in brain tumor and tuber-culous meningitis, 0000123100.

Abnormal Findings.

a. See Table 26 on page 154.
b. A luetic or paretic type curve does not indicate syphilis, unless the Wassermann test is also positive.
c. A luetic type curve is found frequently in fluid in which there is an excess of protein from any cause.
d. A luetic or paretic type of curve may be obtained in myxedema.

II. Preparation of Colloidal Gold.

A. *General Considerations.*

1. Use chemically clean "Pyrex" glassware.
2. Clean again with aqua regia (1 volume HNO_3 to 3 volumes HCl) and rinse thoroughly first with single distilled water then with double distilled water.
3. All chemicals should be Merck's "Blue Label."
4. The solutions should be made in double distilled water and the sodium citrate solution made fresh each time.

B. *Borowskaja's Modification.*

1. Add 10 cc. of a 1% solution of acid yellow gold chloride to 950 cc. of double distilled water in a 2 liter beaker.
2. Heat to 90°C. and add 50 cc. of a 1% solution of sodium citrate.
3. Boil, stirring constantly until a dark-red color appears, then watch carefully looking through the solution to the daylight until a cherry-red color appears without any evidence of blue. The longer the solution boils the more sensitive it becomes.
4. Allow the solution to cool slowly.
5. After the solution has stood for 24 hours, it must be checked with a known positive and negative spinal fluid.

Body Fluids

Transudates and Exudates

I. General Considerations.

A. ***Transudates.***

1. These are fluids which accumulate in serous cavities as a result of noninflammatory processes.
2. Usually due to a disturbance of circulation with passive congestion and edema.
3. Examples are effusions into the pleural, pericardial, and peritoneal cavities.

B. ***Exudates.***

1. These are fluids which accumulate in tissues and serous cavities as a result of an inflammatory process.
2. Usually due to bacterial infection.

C. ***For distinguishing characteristics*** see Table 27.

II. Collection of Fluid.

A. All fluid collected should be sent to the laboratory as soon as possible.

B. If cultures are to be made, the fluid must be collected in a sterile container and kept free from contamination.

C. If cell count and chemical determinations are to be made on exudates, a small amount of lithium oxalate should be added to the fluid immediately after withdrawal.

D. For differentiation of transudates and exudates see Table 27.

III. Chemical Examination.

A. ***Protein.***

1. *Qualitative Test* (Rivalta's Method).
   a. Add 0.1 cc. of glacial acetic acid to 100 cc. of water in a graduated glass cylinder and mix.
   b. Allow a drop of fluid, free from cells, to fall into this solution.
   c. Normal body fluids give no cloud, transudates give only a slight cloud, and inflammatory exudates give a heavy precipitate, often falling to the bottom with the drop of fluid.
   d. The precipitate is produced by serosamucin.
2. *Quantitative Test.*
   a. Use same method as for serum total protein, page 275, except make a 1-25 dilution instead of a 1-50.
   b. See Table 27 for interpretation.

B. ***For other chemical substances,*** follow directions for determination for that particular substance in blood.

1. Most nonprotein substances in exudates and transudates parallel their values in the blood plasma.
2. Uric acid is higher in transudates from tumor cases.
3. Cholesterol varies considerably; it is high in chylous and chyloid exudates.

IV. Microscopic Examination.

A. ***A cell count*** may be made by the same method used for spinal fluid except use 0.85% NaCl solution instead of the diluting fluid described.

B. ***Differential Count.***

1. Place fluid in a 50 cc. centrifuge tube and centrifuge at a high rate of speed for 30 minutes. Centrifugation for tumor cells will be described later.
2. Pour off the supernatant fluid and make a smear of the sediment.
3. Make a Wright's stain and differentiate 100 cells.

C. ***Smear for Bacteria.***

1. Centrifuge as described under differential count.
2. For bacteria make a smear, dry, fix, and stain by Gram's method.
3. For tubercle bacilli make a smear, dry, fix, and stain by Ziehl-Neelsen method.

D. ***Examination for Tumor Cells.***

1. The entire amount of fluid must be centrifuged as soon as possible after removal.
2. Fill four to eight 50 cc. centrifuge tubes and centrifuge at a high rate of speed for 30 minutes.
3. Pour off the supernatant fluid, add more fluid to the sediment, and centrifuge again.
4. Repeat until all the fluid is centrifuged.

Wash sediment from all tubes but one with 10% formalin into the one tube.

Centrifuge and pour off the supernatant fluid.

Melt a tube of plain agar and pour into a mold.

Loosen the sediment in the bottom of the test tube and pour directly into the agar in the mold. The sediment will settle to the bottom of the agar.

Cool in the refrigerator and trim off the excess agar.

Fix the block of agar in 10% formalin for 24 hours.

Dehydrate the agar block as for tissues, embed in paraffin, cut, and stain with hematoxylin and eosin.

TABLE 27. DIFFERENTIATION OF TRANSUDATES AND EXUDATES

	Transudates	Exudates
pearance	Clear, serous, light yellow.	Clear or cloudy. Serous, flocculent (fibrin), purulent, hemorrhagic, chylous, chyloid, or any combination.
ecific gravity	Below 1.018.	Above 1.018.
ot	None.	Clots spontaneously.
otein	Less than 2.5 gm.%.	More than 2.5 gm.%.
rosa-mucin Rivalta Test)	Negative.	Positive.
ells	Few endothelial cells and small lymphocytes. Tumor cells may be found. Eosinophils are increased after repeated tappings.	Many polymorphonuclears in acute infection. Small lymphocytes in chronic infections. Erythrocytes usually present.
acteria	Absent.	Usually present.

Synovial Fluid

Synovial Fluid is obtained by aspiration of a joint, bursa, or tendon sheath.

II. Cell Count.

Method.

Use the same method as described under spinal fluid except use 0.85% NaCl solution instead of diluting fluid described.

Normally there are 50 to 60 leukocytes per c. mm.

Differential Count.

Centrifuge some of the fluid at high speed for 30 minutes, pour off the supernatant fluid, and make a smear of the sediment.

When dry make a Wright's stain and differentiate 100 cells.

Normal differential count reveals 60 to 70% monocytes, 20 to 30% lymphocytes, 5 to 10% neutrophils, and 4% synovial cells.

III. Bacteriology.

A. ***Direct Smear.***

1. Centrifuge as described under differential count.
2. Make a smear of the sediment, dry, fix, and make a Gram stain.
3. Examine for bacteria.

B. ***Cultures.***

1. See Section on Bacteriology, page 183.
2. Culture for N. gonorrhoeae as described on page 182.

C. ***Complement-fixation Test.***

1. To rule out syphilis use synovial fluid in place of serum in the routine method for the Wassermann test, see pages 226 and 232.
2. To rule out gonorrhea use synovial fluid instead of serum in the gonococcus complement-fixation test, see pages 228 and 234.

IV. Abnormal Findings.

A. ***Chronic Infective Arthritis.***

1. Leukocytes are increased to 10,000 or more per c. mm.
2. Cultures are usually positive.
3. In gonorrheal arthritis there may be a positive gonococcus complement-fixation test.

B. ***Syphilitic Arthritis.***

1. If the complement-fixation test for syphilis is positive and the blood test is negative, there is definite evidence the joint lesion is due to syphilis.
2. If the blood test is positive as well as the synovial fluid, there is no evidence the joint lesion is due to syphilis.

C. ***Trauma***—there may be many erythrocytes.

Semen

I. Macroscopic Examination.

A. *Volume.*

1. Measure the volume in a small graduated cylinder.
2. The amount varies between a few drops up to 10 cc.; samples less than 1.5 cc. are considered below normal.

B. *Viscosity.*

1. Freshly ejaculated semen has a high degree of viscosity.
2. Self-liquefaction should be complete after 30 minutes.

C. *Reaction*—pH.

1. It is always alkaline having a range of 7.2 to 8.9, with an average of 7.8.
2. The reaction of semen has little significance.

II. Spermatozoa.

A. *Motility.*

1. After ejaculation the semen should be transferred immediately from the condom to a clean bottle or test tube, as the spermicidal substances in the condom will cause cessation of motility of the spermatozoa.
2. Place a large drop of semen on a slide, cover with a cover glass, and rim with vaseline.
3. Examine with the high dry objective of the microscope.
4. Note roughly the proportion of motile to nonmotile cells and also look for testicular cells, epithelial cells, leukocytes, erythrocytes, and crystals.
5. Examine the slide after 3, 6, 12, and 24 hours to determine when motility ceases.
6. There should be no reduction of activity 3 hours after emission, slight reduction after 6 hours, and usually complete cessation of motility after 12 hours at room temperature.

B. *Counting of Spermatozoa.*

1. Mix the specimen thoroughly by shaking gently.
2. Use a leukocyte counting pipette and draw the semen up to the 0.5 mark, then dilute to mark 11 with the following diluting fluid.

Sodium bicarbonate	5 gm.
Formalin (neutral)	1 cc.
Distilled water	100 cc.

3. Place on a hemocytometer counting chamber in the manner as for a leukocyte count, allow cells to settle for 2 minutes, and count the cells in 4 sq. mm.
4. *Calculation:*
   a. Obtain the total number of spermatozoa in 1 c. mm. of semen by multiplying the number counted in 4 sq. mm. by 50.
   b. The results are reported in cc.; therefore, the number per c. mm. is multiplied by 1000.
5. *Normal Count:* 100–150 million per cc.

C. *Stained Smear.*

1. After spontaneous liquefaction of the semen, prepare a thin smear on a clean glass slide; the smear should be made within 2 hours.
2. Dry in air and fix gently with heat by passing the slide through a flame.
3. When the slide has cooled to room temperature, cover with a 0.25% aqueous solution of basic fuchsin and stain for 5 minutes.
4. Wash with tap water, dry, and examine under the oil immersion objective of the microscope.
5. The heads of the spermatozoa are a dark red color while the tail and middle piece are pink.
6. Count the number of spermatozoa in a microscopic field without regard to their morphology.
7. Search the same field for immature forms of spermatozoa.
8. Examine 100 to 500 spermatozoa for abnormalities.
   a. Heads—too small, too large, pointed, ragged edges, atypical distribution of chromatin, vacuoles, or double heads.
   b. Middle piece—absent, bifurcated, swollen.
   c. Tails—double, curled, rudimentary, or absent.
9. Report per cent of abnormal forms and immature forms; also report the presence of epithelial cells, erythrocytes, and leukocytes.
10. At least 80 per cent should be normally formed.

Pregnancy Tests

I. Friedman Test.

Principle: An anterior-pituitary-like hormone is produced in pregnancy by the placenta and excreted in the urine. When urine containing this hormone is injected into virgin female rabbits it produces ripening of and hemorrhage into the follicles of the ovaries.

Method.

The patient should be instructed to drink very little water the evening before the test. The patient should not take any aspirin, quinine, or barbiturates for at least 24 hours before collecting the urine as these drugs will kill the rabbit.

Collect the first morning specimen of urine in a clean container and keep in a cool place to avoid bacterial growth.

If the urine is not acid, add 10% acetic acid one drop at a time until acid to nitrazine paper. The hormone is more active in an acid medium (pH 6.5-7.0).

Take the specific gravity and if it is less than 1.020, save a portion of the urine for a second injection of 10 cc. the next day. Filter and warm the urine to body temperature.

Inject 15 to 20 cc. slowly into the marginal ear vein of a female rabbit; sterile precautions are not necessary.

The rabbit should be not less than 17 weeks old and should weigh over 4 pounds. It must be kept isolated from all other rabbits for 8 to 9 weeks before the test.

Kill or operate upon the rabbit in 30 to 48 hours after the injection and examine the ovaries.

If negative, the ovaries are pure white or light pink and large mature follicles may be present.

False negative reactions may be due to the use of rabbits not old enough to produce a positive reaction. In these rabbits the ovaries are narrow, flat, have an opaque appearance, and the uterine horns are pure white.

If positive, from 1 to 14 corpora hemorrhagica and corpora lutea are present in each ovary, see Fig. 19.

13. *Positive reactions* occur in the following:
    a. In 95-97% of normal pregnancies a positive reaction may be obtained as early as 5 to 14 days after conception, but negative tests are not dependable until 10 days after the first missed period.
    b. In about 50% of ectopic pregnancies; these are followed by negative results in 3 weeks after the onset of vaginal bleeding.
    c. After fetal death, missed or incomplete abortions, as long as living placental tissue is present.
    d. In hydatidiform moles and chorionepithelioma.
    e. In men with choriocarcinoma and sometimes with teratoma and embryonal carcinoma of the testicles.

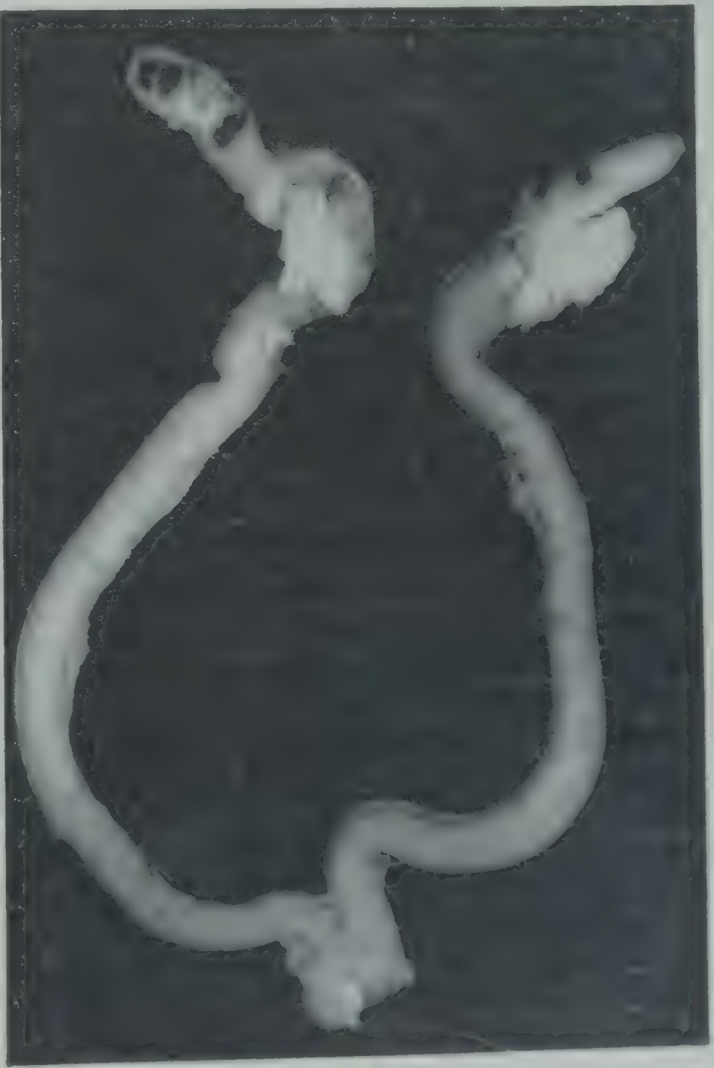


Fig. 19. Rabbit Ovaries Showing Corpora Hemorrhagica and Corpora Lutea in a Positive Friedman Test

   f. In tumors of the pituitary in which there is an increased production of gonadotropic hormones.

C. *Quantitative Friedman Test.*

1. This test may be used in suspected cases of hydatidiform moles, chorionepithelioma, and tumors of the testes.
2. Make a 1-10 and a 1-100 dilution of urine in 0.85% NaCl solution.
3. Inject 1 rabbit with 5 cc. of the 1-10 dilution which equals 0.5 cc. of undiluted urine.
4. Inject another rabbit with 5 cc. of the 1-100 dilution which equals 0.05 cc. of undiluted urine.
5. If the rabbit injected with the 1-100 dilution is strongly positive in 48 hours, inject a third rabbit with 5 cc. of a 1-1000 dilution which equals 0.005 cc. of undiluted urine.
6. In cases of hydatidiform moles, chorionepithelioma, and certain tumors of the testes, the pituitary-like hormone is present in a much higher concentration than in cases of pregnancy.
   a. Occasionally 0.5 cc. of urine from a pregnant woman will give a positive reaction.
   b. A positive reaction with 0.05 cc. of urine is almost certainly to be due to one of the above mentioned tumors.

II. Hoffmann Test.

A. *Method.*

1. Obtain about 12 cc. of venous blood, allow to clot, centrifuge, and separate the serum.
2. The same precautions about medication should be taken as when urine is used.
3. Inject 3 to 4 cc. of the serum into the marginal ear vein of a female rabbit similar to that used in the Friedman Test.
4. Kill or operate upon the rabbit 30 to 48 hours after the injection.

B. ***Findings*** are identical with those in the Friedman test. This method is useful when the urine contains a toxic substance which kills the rabbit.

III. Quantitative Ascheim-Zondek Test.

A. *General Considerations.*

1. Six immature female mice weighing from 5 to 8 grams are necessary for the test; three are inoculated with unconcentrated urine and three with concentrated urine.
2. A 24 hour specimen of urine is necessary; if alkaline, acidify with a few drops of glacial acetic acid.

B. *Unconcentrated Urine.*

1. Inject each of 3 mice underneath the skin of the back with 0.1, 0.2, and 0.4 cc. of urine respectively.
2. Repeat injections 4 times during the next 48 hours, keeping urine in the refrigerator in the meantime.
3. Kill the mice with ether 100 hours after the last injection.
4. Examine the ovaries with a hand lens and then place in 10% formalin for sections.
5. Normal ovaries are pinhead in size and pale in appearance.
6. *Positive reactions* consist of:
   a. Reaction I—hyperemia and swelling of the Graafian follicles together with formation of cumulus oophorus.
   b. Reaction II—hemorrhages found in the ripened follicles.
7. Positive reactions in the different mice are equivalent to 2000 (0.1 cc.), 1000 (0.2 cc.), and 500 (0.4 cc.) mouse units of hormone per liter.

C. *Concentrated Urine.*

1. To 40 cc. of urine, add 200 cc. of 95% alcohol and let stand overnight in the refrigerator. A precipitate containing the hormone will settle to the bottom.
2. Next day siphon off the supernatant fluid, leaving the precipitate with about 40 to 45 cc. of fluid.
3. Mix the precipitate with the fluid and pour into a centrifuge tube.
4. Centrifuge for 5 minutes; then pour off the supernatant fluid.
5. Add about 30 cc. of ether to the precipitate and stir for 10 minutes.
6. Centrifuge for 5 minutes and then pour off the ether.
7. Spread the precipitate around the bottom of the tube with a glass rod and allow to dry.
8. When dry, add 8 cc. of distilled water and let stand overnight.
9. Next day centrifuge and pour the clear supernatant fluid containing the hormone into a clean test tube. Keep in the refrigerator.
10. Inject 3 mice with 0.1, 0.2, and 0.4 cc. of this supernatant fluid as directed under B above.
11. Positive reactions in the different mice are equivalent to 400 (0.1 cc.), 200 (0.2 cc.), and 100 (0.4 cc.) mouse units of hormone per liter.

Bacteriology

General Considerations

. Bacteriology has many clinical applications in medicine.

- The isolation and identification of bacteria to determine the causative agent in infections.
- The identification of specific types in mixed species, e.g., the types of pneumococci and streptococci.
- The preparation of antigens for diagnostic agglutination tests.
- The preparation of autogenous vaccines.

. Diagnosis can be made by 5 general methods in clinical bacteriology.

- Morphology on a direct smear.
- Culture on various types of media.
- Demonstration of antigenic properties, such as typing pneumococci, meningococci, etc.
- Demonstration of antibodies as in agglutination tests.
- Animal inoculation.

. Certain rules should be so well known that they may be applied almost automatically:

- The strictest aseptic precautions must be exercised in the securing of material for bacterial study, e.g., urine should be obtained by sterile catheter.
- Cultures must be protected from contamination not only at the time they are made but while they are being examined or subcultured. Air currents and dust must be particularly avoided.
- Infections of laboratory workers are not infrequent. To avoid these, observe the following:

1. When a culture is accidently spilled on a table or floor, it must be immediately disinfected by strong antiseptic solutions, such as phenol, formalin, or bichloride of mercury. Allow the disinfectant to remain in contact with the spilled culture for at least one-half hour before cleaning up the debris.
2. Exercise care in sterilizing platinum needles and loops to avoid spattering. This is a common source of accidental infections.
3. Material from living cultures must not be blown from pipettes in order to prevent contaminating surrounding area.
4. Pipettes used in handling or transferring living cultures must be placed immediately in disinfecting solutions. Capillary pipettes that are not to be used again may be sterilized by heating to redness (with precautions against spattering) before being discarded.

D. Certain bacteria are so dangerous that they should not be kept in ordinary hospital laboratories. These include the organisms of bubonic plague, glanders, brucellosis, and tularemia.

E. Some bacteria require a special type of media for growth. The order by the clinician for a bacteriologic study of any case should, therefore, indicate the general type of infection suspected in order that the bacteriologist may select the proper media for making cultures. Otherwise a wide variety of media will have to be used and both material and time will be wasted.

Smears

I. Purpose of Smears.

A. Determination of the morphology of bacteria—cocci, diplococci, bacilli, spirilla, spirochetes, spores, capsules, flagella, etc.

B. Determination of the staining affinity of bacteria—gram-positive, gram-negative, acid-fast, etc.

C. Demonstration of the purity of cultures.

D. Demonstration of bacteria present in material to be cultured, as a check on types that grow in cultures and for choice of culture media.

E. Diagnosis by direct smear—organisms of Vincent's angina, gonococci, tubercle bacilli, etc.

II. Preparation of Smears.

A. ***Smears of Material Obtained from Patient.***

1. At least 2 smears should be prepared from each specimen.
2. The slides must be clean and not scratched.
3. The smears should be made with sterile cotton swabs or with flamed stiff wire loops.
4. The smears must not be so thick that pus cells are piled up on each other.
5. They must not be too thin or false negative results may be reported.
6. The swab should be rolled on the slide and should not cover the same area twice.

7. Material poor in cells may be first centrifuged and smears prepared of the sediment.
8. Label and date all slides on which smears are made.

B. *Smears of Growth on Culture Media*—see identification of bacteria under cultures, page 169.

C. *Fixing Smears.*

1. Dry in air (smears of urine sediment should dry in the incubator for 3 hours or overnight at room temperature).
2. Fix by passing slide quickly through a flame 2 or 3 times.
3. The slide should only feel warm but not hot to the skin when placed on the back of the hand.

Stains

Refer to Section on Solutions used in Routine Tests (p. 359) for method of preparing staining solutions.

I. Gram Stain.

A. *Hucker's Modification.*

1. Stain preparation 1 minute with crystal violet solution.
2. Wash with water.
3. Cover with Gram's iodine solution for 1 minute.
4. Wash with water.
5. Decolorize with 95% alcohol until no more color dissolves out.
6. Wash with water.
7. Counterstain with safranin solution for 1 minute.
8. Wash with water and dry.
9. Dilute carbol-fuchsin solution (Ziehl-Neelsen stain diluted 1-20 with water) may be used as a counterstain instead of safranin.

B. *Gram-positive organisms* are stained deep violet, almost black.

1. *Cocci.*
    Diplococcus pneumoniae
    Gaffkya tetragena
    Sarcina group
    Staphylococcus group
    Streptococcus group
2. *Bacilli.*
    Bacillus anthracis
    Bacillus subtilis
    Clostridium group (see Table 30)
    Corynebacterium diphtheriae
    Corynebacterium pseudodiphtheriticum (C. hoffmannii)
    Corynebacterium xerose
    Lactobacillus acidophilus (B. Boas-Oppler)
    Mycobacterium leprae
    Mycobacterium smegmatis
    Mycobacterium tuberculosis
3. *Fungi.*
    Candida albicans
    Mycelium of actinomyces
    Saccharomyces

C. *Gram-negative organisms* are stained red.

1. *Cocci.*
    Neisseria group (see Table 34)
2. *Bacilli.*
    Aerobacter aerogenes
    Borrelia vincentii (S. vincenti)
    Brucella abortus
    Brucella melitensis
    Brucella suis
    Escherichia coli
    Fusobacterium plauti-vincenti (B. fusiformis
    Hemophilus ducreyi
    Hemophilus hemolyticus
    Hemophilus influenzae
    Hemophilus pertussis
    Klebsiella pneumoniae (B. friedländeri)
    Moraxella lacunata (B. Morax-Axenfeld)
    Pasteurella pestis
    Pasteurella tularensis
    Proteus group (see Table 33)
    Pseudomonas aeruginosa (B. pyocyaneus)
    Salmonella group (see Table 33)
    Shigella group (see Table 33)
    Vibrio comma (S. cholerae)

II. Methylene Blue Stain.

A. *Method.*

1. Cover preparation with Loeffler's methylene blue solution for 1 or 2 minutes.
2. Wash with water and dry.

B. *Used* mostly for C. diphtheriae and fungi.

III. Ziehl-Neelsen Stain.

A. *Method.*

1. Apply as much carbol-fuchsin solution to the preparation as will stay on the slide.
2. Steam (do not boil) over a flame for 5 minutes, replacing stain as it evaporates.
3. Let the slide cool until a sheen appears on the stain.
4. Wash with water and decolorize with acid alcohol (3 cc. conc. HCl in 97 cc. of 95% alcohol) until all the pink disappears from the thinner portions of the smear.
5. Wash and counterstain with Loeffler's methylene blue solution for 1 minute.
6. When decolorizing smears made from urine sediment or feces, use acid alcohol made with nitric acid (5 cc. conc. nitric acid in 95 cc. of 95% alcohol).

id-fast bacilli retain the red stain while all
er bacteria stain blue.

tain for Vincent's Angina Organisms.

thod.

in preparation 1 or 2 seconds with carbol-
hsin solution (stain used in the Ziehl-
elsen method).
ash with water and dry.

ndings.

amine for fusiform bacilli and spirochetes.
th must be found in considerable numbers
fore giving a positive diagnosis for Vin-
nt's angina.
ese organisms are anaerobic and do not
ow on an ordinary throat culture.

V. Special Stains.

ailey's Flagella Stain.

ace a large drop of distilled water on a warm
de, add a small loopful of organisms from
18-24 hour agar culture, and mix gently
order not to detach the flagella.
nmediately tilt the slide so the drop runs
own the slide making a thin film; it is im-
ortant the slide dry quickly.
over with mordant for 2 minutes.
our off mordant and cover with mixture for
agella stain for 7 minutes.
Vash with water.
over with Ziehl-Neelsen carbol-fuchsin solu-
on and gently steam for 30 seconds.
Vash with water and drain dry by standing
e slide on filter paper.
he flagella stain red and are arranged on
e bacterial body in various positions.
A single flagellum at one pole (Monotricha).
A tuft of flagella at one pole (Lophotricha).
Flagella at both ends (Amphitricha).
Flagella completely surrounding the bacterial body (Peritricha).

lordant.

Mix 3 parts of 5% tannic acid solution with 1 part of 10% ferric chloride solution.
Keeps indefinitely but must be freshly filtered before using.

lixture for Flagella Stain.

Must be made fresh just before using.
Place 7 drops of mordant in a small test tube, add 1 drop of Ziehl-Neelsen carbolfuchsin solution and mix.
Add 1 drop of conc. HCl and mix.
Add 1 drop of formalin and mix.

B. *Bailey's Capsule Stain.*

1. Make a thin smear of the exudate or add a small loopful of an 18-24 hour culture to a drop of distilled water on a slide.
2. Air dry; do not heat.
3. Cover with mordant for 15 seconds. (See Bailey's flagella stain for mordant.)
4. Wash with water.
5. Stain with dilute carbol-fuchsin solution for 10 seconds (1 part Ziehl-Neelsen carbolfuchsin solution to 9 parts of distilled water).
6. Wash with water and dry.
7. Capsule stains pink and the bacterial body deep red.

C. *India Ink Capsule Stain.*

1. Place a drop of 6% glucose solution near one end of a perfectly clean slide and add a loopful of organisms to make a thin suspension.
2. Mix a small drop of India ink with the suspension and with the edge of another slide spread the suspension over the slide so as to produce a thin even gray smear.
3. Air dry; do not heat smear.
4. Stain for 5 minutes with safranin solution and then wash with water.
5. Allow to dry; *do not blot.*
6. If examining exudates, mix ink with exudate and then add a drop of glucose solution and spread quickly.
7. The bacterial body stains red and the capsule is colorless against a black background.

D. *Spore Stain.*

1. Place a drop of distilled water on a slide, add a small loopful of organisms, mix, and spread out in a thin smear.
2. Cover with Ziehl-Neelsen carbol-fuchsin solution and steam for 5 minutes.
3. Decolorize with 95% alcohol for 2 minutes.
4. Counterstain with Loeffler's methylene blue solution for 1 minute.
5. The spores appear red and the bacterial bodies (vegetative forms) blue.
6. The spore may be central, subterminal, or terminal in the cell.

E. *Tilden's Stain for Spirochetes.*

1. Place a drop of buffered formalin on a slide, add a drop of exudate, and mix gently.
2. Spread out on the slide to make a thin smear and dry.
3. Stain 2 or 3 minutes with the crystal violet stain used in the Gram stain, wash with tap water, and dry.
4. *Buffered Formalin.*

Formalin	1 part
M/15 phosphate buffer (pH 7.6)*	9 parts

*See Table 79, Page 260.

TABLE 28. CHOICE OF CULTURE MEDIA FOR DIFFERENT MATERIALS

Source of Material	Broth		Glucose Agar Slant	Agar Plate			Loeffler's Medium	Special Cultures if Suspected
	Glucose	Thioglycollate		Chocolate	Blood	E.M.B.		
Exudates (Wounds, abscesses, ulcers, etc.)	+	+	+		+			Tularemia Tetanus Gas Bacillus Fungus
Eye	+		+	+	+		+	Tularemia
Ear	+	+	+		+			Fungus
Nose and Throat	+	+	+		+		+	Pertussis
Sputum	+	+	+		+			Tuberculosis Fungus
Urine	+	+	+		+	+		Tuberculosis
Feces						+		Gas Bacillus Streptococci
Genitalia	+	+		+	+	+		Fungus
Spinal fluid	+	+	+		+			Meningococcus
Blood*	100 cc. plain broth	+	100 cc. plain agar					Brucella Tularemia Pneumonia
Transudates	+	+	+		+			
Postmortem material	+	+	+		+			

*100 cc. of tryptose broth.

Cultures

I. General Rules for Making Cultures.

A. ***Collection of Specimen.***

1. Material for cultures must be taken aseptically, placed in a sterile container, and kept free from contamination.
2. Material to be cultured should not come in direct contact with disinfectants.
3. If anaerobic cultures are to be made, preserve anaerobic bacteria by inoculating some of the specimen in thioglycollate glucose broth at once.
4. All material should be cultured as soon as possible after collection from source.
5. All specimens must be labelled and dated.

B. ***Preparation for Culturing.***

1. Close all windows.
2. Sponge table with 5% lysol solution first thing in the morning.
3. Media for culture should be chosen in accordance with the source of material and the organisms expected. See Table 28.
4. Liquids are centrifuged and the sediment cultured.
5. Thick pus, mucus, etc., must be thinned by adding broth; the amount added depending on the amount and thickness of the material.
6. Swabs with the material to be cultured are placed in tubes containing 2 cc. of glucose broth.
7. Save material and cultures from which transfers are made until satisfactory growth is obtained.
8. If cultures can not be made immediately place all materials except spinal fluid in the refrigerator. Spinal fluid *must* be kept in the incubator but should be cultured immediately.

C. ***Media.***

1. Use culture media best suited to the organisms suspected.
2. Warm media to body temperature before inoculating.
3. If the patient has been receiving sulfonamide therapy, the media should contain 5 mg. of para-aminobenzoic acid per 100 cc.
4. If the patient has been receiving penicillin therapy, penicillinase should be added to the media.

Penicillinase may be obtained in powder form from the Schenley Laboratories, Inc., 350 Fifth Ave., New York 1, N. Y.

Sterilize the cap of the vial with alcohol or an antiseptic solution.

Inject 10 cc. of sterile distilled water into the vial using a syringe and needle that has been boiled or autoclaved (chemical agents have a deleterious effect upon penicillinase).

This working solution contains 100 units of penicillinase per cc. and will keep 3 months in the refrigerator.

For each 15 cc. of medium, broth or melted agar cooled to 45°C., add 0.1 cc. (10 units) of the working solution.

The working solution may be added to a large amount of media and stored in the refrigerator.

he dehydrated culture media made by Difco boratories of Detroit and the Baltimore ological Laboratory (BBL) of Baltimore are tisfactory and save a great deal of time in e laboratory. The directions for preparing ch medium is given on the package so will t be repeated here.

ood Agar.

Obtain at least 10 cc. of human, horse, sheep, or rabbit blood aseptically and defibrinate by placing in a sterile flask containing glass beads or oxalate and then shaking. (If glass beads are used, the flask must be shaken for 5 minutes.)

. Melt 100 cc. of sterile beef infusion agar and cool to 45°C.

Add 5 cc. of the defibrinated blood aseptically and mix gently.

. Pour aseptically into sterile Petri dishes or tubes.

hioglycollate glucose broth (Brewer's anerobic medium) is obtained from the Baltiore Biological Laboratory and made accordg to directions on the package.

. No para-aminobenzoic acid need be added to this medium.

. Store at *room temperature.*

. It supports growth of both facultative and strict anaerobes.

. Inoculate with a large amount of material and rotate gently so that the culture is mixed thoroughly.

. Incubate aerobically.

. Shake culture well before making smears or subcultures, as organisms are apt to grow in colony formation in the depths of the medium.

. Subculture on two blood agar plates; incubate one anaerobically and the other aerobically.

8. *Sugar Media for Identification.*
   a. The following sugars (1 gm. to 100 cc. of medium) are added to beef extract (not infusion) broth containing 1 cc. of Andrade's indicator in 100 cc. of medium and autoclaved between 10 and 12 lbs. pressure for 15 minutes.
      Glucose
      Mannitol
      Sucrose
      Lactose
   b. The following sugars are used in a 5% aqueous solution which has been passed through a Seitz filter while warm and then added to autoclaved medium (containing an indicator) in the proportion of 1 cc. to 10 cc. of broth.
      Inulin
      Dulcitol
      Dextrin
   c. The following sugars are used in a 10% aqueous solution which has been passed through a Seitz filter while warm and then added to the autoclaved medium (containing an indicator) in the proportion of 0.5 cc. to 10 cc. of broth.
      Maltose
      Rhamnose
      Raffinose
      Inosite
      Arabinose }
      Levulose } Will filter when cold.
      Xylose }
   d. For the study of gas formation, use Dunham's fermentation tubes which consist of standard culture tubes with small tubes (8 by 25 mm.) inverted inside them. When the medium is autoclaved, the inverted tube becomes filled with the medium. Any gas produced in cultures is trapped in the inverted tube. Sealing of the fermentation tubes with paraffin coated corks often increases the rapidity with which a sugar is attacked by bacteria.
   e. For the identification of streptococci, pneumococci, etc., add 0.2 per cent agar to the broth. (This cannot be used for the study of gas formation.)

9. *Greenspoon's Modification of Loeffler's Blood Serum.*
   a. Mix 1 part of 1% glucose bouillon with 3 parts of blood serum.
   b. Add 1 cc. of a 50% sodium citrate solution to each 100 cc. of the above mixture and adjust the reaction to pH 6.4 with a

3% citric acid solution using bromthymol blue as an indicator.

c. Autoclave as described under culture medium for tubercle bacilli (p. 179).

d. Gram-positive cocci are inhibited by this medium.

D. *Inoculating Media.*

1. *General Considerations.*
   - a. A platinum or nichrome wire clamped in a needle holder is used to inoculate media.
     - 1) One wire should have a loop 2 mm. in diameter for streaking solid media, another wire a loop 5 mm. in diameter for inoculating fluid media, and another left straight for making stab cultures.
     - 2) The wire must be heated to redness before and after each inoculation.
     - 3) It must be cooled before placing into the material to be cultured.
     - 4) It must be passed in and out of the culture tubes without touching the side.
   - b. The mouths of all tubes containing media or material to be cultured must be flamed after removing cotton plugs and before replacing them.
   - c. When many bacteria are expected, only a small amount of material is used and streaked out for isolation.
   - d. When only a few organisms are expected, a larger quantity of material is used.
   - e. Pieces of tissue for culturing are placed in a sterile mortar containing sterile sand and sterile 0.85% NaCl solution and are ground with a sterile pestle. The supernatant fluid is used for the inoculation.
   - f. Label all culture tubes plainly with the patient's name and the date.
2. *Inoculating Plates.*
   - a. Use a 2 mm. wire loop which has been bent slightly so that the loop may be held parallel with the surface of the agar.
   - b. *Method I.*
     - 1) Streak a loopful of material back and forth at the upper edge of the plate.
     - 2) Flame the loop and cool.
     - 3) Run the loop through the previously streaked area several times and then streak the top half of the plate in closely parallel lines.
     - 4) Turn plate half way around and streak the other half upward by holding the inoculating needle up so that only the edge of the loop touches the agar.
   - c. *Method II*—refer to Fig. 20.
     - 1) Streak a loopful of material back and forth in closely placed parallel lines over one half the surface of the medium in the plate (A).
     - 2) Flame the loop, turn plate at a righ angle, and then streak one half of th uninoculated surface overlapping th original streak not more than 1 cm (B).
     - 3) Flame the loop, turn the plate agai at a right angle, and streak the remaining surface of the plate overlapping a portion of the preceding section a before (C).

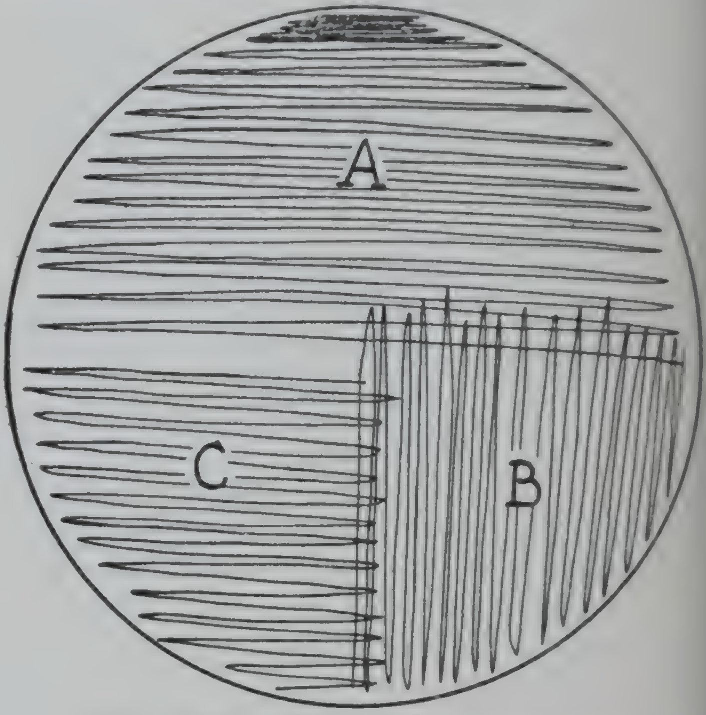


FIG. 20. METHOD II FOR INOCULATING PLATES.

3. *Pour Plates.*
   - a. Dilute the material to be cultured in 10 cc. of broth.
   - b. Melt 10 cc. of agar and cool to 45°C.
   - c. Add 0.1 to 0.5 cc. (depending on the number of organisms present) of the mixture and mix by rolling the tube between the palms of the hands.
   - d. Pour into a Petri dish to solidify.
4. *Stab Culture.*
   - a. Use a straight wire for transferring some of the organisms.
   - b. Plunge the wire straight downward into the unslanted agar or into the gelatin.
5. *Shake Culture.*
   - a. Melt 10 cc. of agar in a tube and cool to 45°C.
   - b. Inoculate with one loopful of organisms and mix by rotating the tube between the palms of the hands.
   - c. Leave at room temperature until solidified.

E. *Incubation.*

1. Cultures for aerobic organisms growing best at body temperature are placed in an incubator which maintains a uniform temperature of 37°C.

Cultures for anaerobic organisms are placed in an anaerobe jar and incubated at 37°C. for 48 hours before opening the jar.

a. A museum jar having a screw clamp cover and a capacity of 8 pints is used.
b. A small, flat metal holder containing about 5 gm. of calcium carbonate is placed in the bottom of the jar.
c. Plasticine is placed on the ground edge of the glass jar.
d. Place cultures in the jar.
e. Using forceps, place a piece of yellow phosphorus the size of a grain of rice on the calcium carbonate.
f. The lid is pressed down onto the plasticine immediately to make the jar airtight. The lid is held in place by the screw clamp.
g. After a few minutes the phosphorus ignites and burns; in this way the oxygen in the jar is exhausted.

Cultures to be grown in an atmosphere of 10% carbon dioxide are placed in a CO_2 jar and incubated at 37°C.

a. A jar similar to the anaerobe jar or a 5 lb. coffee jar with a screw lid and an opening large enough for a Petri dish is used.
b. Place a candle on top of the Petri dishes so that the CO_2 will be diffused throughout the jar as it is heavier than air.
c. Light the candle and screw the top on tightly.
d. When the candle goes out, the jar will contain about 10% CO_2.

Cultures for fungi are grown at room and incubator (37°C.) temperatures.

Gelatin cultures are best incubated at room temperature in order that the type of liquefaction may be observed; if incubated at 37°C. they must be placed in cold water to see if the gelatin will solidify.

Examination of Cultures.

Most cultures are examined after 24 hours.

a. Anaerobic cultures are examined after 48 hours.
b. Cultures for gonococci are examined after 24 or 48 hours, depending on the medium used.

Make smears and subcultures from any growth, reincubate the original cultures for at least 48 hours longer, and examine every 24 hours.

Broth Cultures.

a. Report amount of growth (cloudiness) in pluses (1-4).
b. Record whether growth is homogeneous, granular, flocculent, mucoid, or if pellicle on surface or precipitate on bottom.
c. Make a smear and subculture on solid media.

4. *Solid Media.*
   a. With a wax pencil draw a circle on the underside of the Petri dish around one isolated colony of each type and label the circle with a number.
   b. Record the characteristics of each type of colony: dry, moist, mucoid, rough, smooth, wrinkled, translucent, opaque, flat, elevated, shape of edges, spreading, and color.
   c. Make a smear and subculture of each type of colony.
   d. Use Table 29 as an aid in identification of colonies.

G. *Identification of Bacteria.*

1. *Morphology.*
   a. Have a stock supply of new glass slides with control smears on one end (Fig. 21).
      1) Place a loopful of a broth culture of staphylococci (or a saline suspension of growth from an agar slant) on the upper half of the left hand end of the slide.
      2) Place a loopful of a broth culture of E. coli on the lower half of the same end.

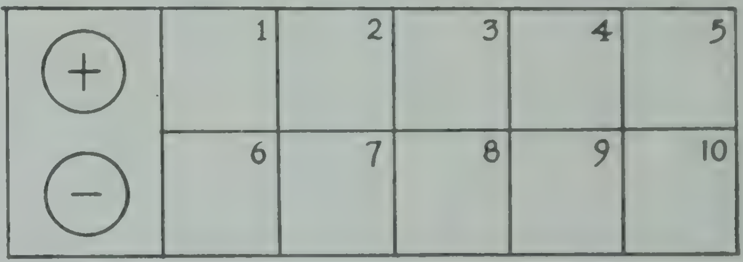


Fig. 21. Microscopic Slide (3 x 1 inch) Marked for Smears and Showing Gram-positive and Gram-negative Controls.

   b. Mark slide in small sections with a wax pencil for smears of unknown cultures (Fig. 21).
      1) Place a loopful of a broth culture in a square.
      2) For smears of colonies on solid media, first place a loopful of distilled water in a square, add a loopful of organisms from the colony, and then mix.
   c. Let the smears dry in air, fix, and stain by Gram's method.
   d. Examine with the oil immersion lens of the microscope for morphology of the bacteria present (see Table 29).
2. *Motility.*
   a. Place a drop of a 24 hour broth culture on a cover glass lying on a horizontal surface.

TABLE 29. APPEARANCE OF ORGANISMS ON BLOOD AGAR AND MORPHOLOGY ON A SMEAR

Gram-positive Cocci

	Type of Colony	Morphology on Smear
Staphylococci	Large, white, lemon, or orange colored, opaque, hemolytic or nonhemolytic.	Cocci in clusters.
Beta Streptococci	Small, white, rather opaque, often quite hard, with a clear zone of hemolysis.	Small round cocci with adjacent sides slightly flattened, occurring in pairs, groups, and very short chains.
Alpha Streptococci	Small, raised, convex, and opaque, surrounded by a narrow zone of green hemolysis.	Vary in size, are elongated cocci, often pleomorphic, occurring in chains.
Gamma Streptococci	Small, gray, and translucent. No hemolysis or slight green discoloration.	Occur in pairs, chains, and small groups, frequently elongated cocci.
D. pneumoniae	Small, flat, shiny, usually transparent with heaped-up center, with green zone surrounding colony. Type III colonies are very large, green, raised, mucoid and confluent.	Lancet-shaped, occur in pairs, sometimes short chains, capsules usually demonstrable.
G. tetragena	Glistening, circular, smooth edges, viscid.	Grouped in tetrads, encapsulated.
Sarcina	Yellow, circular, raised, moist, and glistening.	Grouped in packets of 8 or more cells.

Gram-negative Cocci

N. meningitidis	Fairly large, gray, rather mucoid, and nonhemolytic.	Cocci occurring in pairs with adjacent sides flattened, frequently appear as decolorized staphylococci.
N. catarrhalis	Small, gray, rough, nonhemolytic, often quite hard.	Large cocci in pairs.

Gram-positive Bacilli

Diphtheroids	Small, gray, rather opaque, hard, nonhemolytic with a slightly rough surface.	Short, thick, evenly stained, in palisades and Chinese letter formation.
C. diphtheriae	Small, gray, opaque, usually with a narrow zone of clear hemolysis.	Slender, vary in length, irregular staining. Very pleomorphic, granular, barred and club-shaped. Arranged parallel to each other or at sharp angles to form a V or Y.
B. subtilis	Large, dull, rough, hemolytic with irregular edges.	Large straight rods in chains, easily decolorized.

Gram-negative Bacilli

H. hemolyticus	Soft, pearly, and translucent with large zone of clear hemolysis.	Vary from short rods to long, slender pleomorphic twisted filaments.
H. influenzae	Small, colorless, dew-drop, nonhemolytic, grow out in 1 or 2 days.	Very short, slender, cocco-bacillary forms.
Colon-Typhoid-Paratyphoid and Dysentery Group	Fairly large, gray, translucent, producing clear or green hemolysis, may occasionally be nonhemolytic.	Short plump rods.
Proteus	Thin, shiny, gray film covering the entire surface, usually causing hemolysis.	Vary from rods to long thread-like filaments.
Ps. aeruginosa (B. pyocyaneus)	Large, greenish gray, extremely irregular in shape and presents a stippled appearance.	Similar to Proteus.
K. pneumoniae (Friedländer's)	Large, gray, very soft, mucoid, heaped-up, nonhemolytic, marked tendency to become confluent.	Very short and fat rods, almost coccoid, encapsulated.

- Place a small amount of vaseline on each side of the cavity of a hanging drop slide.
- Turn slide over and press the vaseline down on the edge of the cover glass so that the drop of fluid is in the center of the cavity.
- d. Turn slide over carefully so that the cover glass is right side up.
- Examine with the high dry objective of the microscope.
- Distinguish between true motility and Brownian movement.
- g. Difco Laboratories have a medium to detect motility; however, one must have experience in observing the growth spreading from the stab inoculation.

Subcultures.

Identification of organisms—pure subcultures must be used for the inoculation of sugar media.

Staphylococci.

- a. Inoculate a Loeffler's serum slant for pigment production (report amount): white (albus), lemon (citreus), orange (aureus).
- b. Streak a blood agar plate for hemolysis.
- c. Make a gelatin stab culture and incubate at room temperature.
- d. Inoculate a tube of mannitol broth for fermentation.
- e. Inoculate plain broth for a coagulase test (p. 185).
- f. If the organism is hemolytic and produces pigment, is coagulase positive, ferments mannitol, and liquefies gelatin, it is highly probable that it is a toxin producing organism.

Streptococci.

- a. Inoculate glucose broth for chain formation.
- b. If in doubt whether alpha or beta or gamma types, make pour plates.
  - 1) Inoculate a tube of broth with one loopful of culture and mix.
  - 2) Melt 10 cc. of infusion agar, cool to 45°C., add 0.5 cc. of sterile defibrinated blood and 0.5 cc. of the inoculated broth.
  - 3) Mix by rolling the tube between the palms of the hands.
  - 4) Pour into a Petri dish to solidify.
  - 5) Incubate for 48 hours then place in the refrigerator overnight.
  - 6) A beta type of colony is surrounded by a zone of complete hemolysis; no cells can be seen within this zone when viewed with the low power of the microscope.
  - 7) An alpha type of colony is surrounded by a green discoloration; under low power of the microscope it appears as a zone of incomplete hemolysis.
  - 8) A gamma type of colony produces no change in the surrounding medium.

4. *Pneumococci*—due to their similarity to alpha streptococci, a bile solubility test is necessary for differentiation. See page 184.
5. *Gram-negative bacilli*—inoculate an E.M.B. plate.

II. Isolation of Organisms from a Mixed Culture.

A. *Sodium Azide Medium.*

1. Sodium azide (NaN_3) medium inhibits the growth of both Proteus and other gram-negative organisms.
2. Add 2 cc. of a 1% aqueous solution of sodium azide to 100 cc. of agar medium before autoclaving.
3. When blood is added to the agar, it becomes dark due to hemolysis of the erythrocytes, but this does not interfere with the isolation of beta and alpha streptococci.
   - a. Beta hemolysis appears as a marked greenish discoloration.
   - b. Alpha hemolysis is indicated by a brownish zone around the colony.

B. *Sodium Bicarbonate Medium.*

1. Sodium bicarbonate ($NaHCO_3$) inhibits the growth of gram-negative organisms.
2. Add an equal amount of a sterile 10% aqueous solution of sodium bicarbonate to a 24 hour broth culture and mix by rotating between the palms of the hands.
3. Incubate for 5 hours and then subculture on a blood agar plate.

C. *Alcohol Method.*

1. Alcohol inhibits the spreading of Proteus.
2. Add 5 cc. of 95% alcohol to 100 cc. of blood agar just before pouring into Petri dishes.

D. *Penicillin Method.*

1. Penicillin inhibits the growth of most gram-positive organisms, see Table 37.
2. Add 1 cc. of penicillin solution (1000 units per cc.) to 10 cc. of broth just before inoculating with the mixed culture.
3. Incubate 15 to 18 hours and subculture on a blood agar plate.

III. Culture of Exudate from Wounds.

A. *Routine Cultures.*

1. If pus is thick, thin it by adding a small amount of broth.
2. If material is taken on a swab, place it in

2 cc. of glucose broth.
3. Twirl the swab and press it against the side of the tube when removing, to completely wash out the exudate.
4. Inoculate the following media from the broth.
   a. A blood agar plate, a glucose agar slant, and a tube of thioglycollate glucose broth to be incubated aerobically.
   b. A blood plate and a tube of glucose broth to be incubated in 10% CO_2.
5. Keep the original broth in the refrigerator until satisfactory growth is obtained on subcultures.

B. *Cultures for Cl. perfringens or Cl. tetani.*

1. Heat tubes of sterile milk in boiling water for 30 minutes, cool to 37°C., and then inoculate.
2. Place in an anaerobe jar.
3. The tubes are not heated after inoculation because the organisms are found in the vegetative state in wounds.
4. See Table 30 for differentiation of Clostridia.

C. *Cultures for Fungus*—see Section on Mycology, page 204.

D. *Cultures for P. tularensis*—see blood culture for P. tularensis, page 178.

E. *Organisms Most Likely to be Isolated from Wounds.*

Streptococci
Anaerobic streptococci
Staphylococci
Clostridium perfringens
Diphtheroids
Proteus
Escherichia coli
Bacillus subtilis
Pseudomonas aeruginosa

IV. Culture of Pus from Eyes.

A. *Routine Cultures.*

1. Place swab in a tube containing 1 cc. of Proteose peptone No. 3 broth.
2. From the broth inoculate a glucose agar slant, Loeffler's serum slant, blood agar plate, a chocolate agar plate, and a tube of glucose broth.
3. Incubate the blood agar plate and chocolate agar plate in 10% CO_2, all others aerobically.

B. *Organisms Most Likely to be Isolated.*

Staphylococci
Diphtheroids
Diplococcus pneumoniae
Streptococci
Hemophilus influenzae
Moraxella lacunata (Morax-Axenfeld bacillus)—produces a crater-like digestion of Loeffler's blood serum.
Neisseria gonorrhoeae

V. Ear, Mastoid, and Sinus Cultures.

A. *Routine Cultures.*

1. Place swab in a tube containing 2 cc. glucose broth.
2. From the broth inoculate 2 blood agar plates, one glucose agar slant, one tube of glucose broth, and one tube of thioglycollate glucose broth.
3. Incubate one blood agar plate and the glucose broth in 10% CO_2; incubate the others aerobically.

B. *Cultures for Fungus.*

1. Inoculate material from ear onto two Sabouraud's agar slants.
2. Incubate one at room temperature and one at 37°C.
3. See Section on Mycology for description of Fungi on page 204.

C. *Organisms Most Likely to be Isolated.*

Streptococci
Diplococcus pneumoniae
Proteus
Hemophilus influenzae
Staphylococci
Diphtheroids
Klebsiella pneumoniae
Corynebacterium diphtheriae
Aspergillus
Bacillus subtilis
Coliform group

VI. Throat and Nasopharyngeal Cultures.

A. *Nasopharyngeal Swabs.*

1. Swabs are prepared by tightly wrapping a small piece of cotton about the end of a piece of flexible copper wire (trolling wire) six and a half inches in length.
2. Place swab in a pyrex test tube (5 x 3/8 in.), plug with cotton, and sterilize in the autoclave.
3. To obtain material for a culture, pass the swab back through the nares (only one side necessary) until it touches the posterior nasopharyngeal wall.
4. For the detection of meningococcus carriers, these swabs are streaked on blood agar plates and incubated in 10% CO_2.
5. For detecting whooping cough, see culture for H. pertussis on page 175.

TABLE 30. DIFFERENTIATION OF CLOSTRIDIA

	Spores	Motility	Iron litmus milk*	Lead acetate	Liquefaction of gelatin	Glucose	Lactose	Sucrose	Salicin	Nitrite	Indole	Pathogenicity for guinea pigs
bifermentans	O, S	+	D, B	B	+	AG	—	—	AG±	—	+	—
botulinum	O, S	+	A	B	+	AG	—	—	—	—	—	+
parabotuli-um	O, S	+	A, D, B	—	+	AG	—	—	AG	—	—	+
butyricum	O, S	+	A, C, G	—	—	AG	AG	AG	AG	—	—	—
eseri (hauvoei)	O, S	+	A, Late C	B	+	AG	AG	AG	—	+	—	+
histolyticum	O, S	+	D, B	—	+	—	—	—	—	—	—	+
novyi (oedematiens)	O, S	+	Late C	B	+	AG	—	—	—	—	—	+
perfringens (welchii)	O, S rare	—	A, C, G	B	+	AG	AG	AG	—	+	—	+
putrificum	R, T	+	—	—	+	—	—	—	—	—	±	—
septicum	O, S	+	A, Late C	B	+	AG	AG	—	AG	+	—	+
tertium	O, T	+	A, C, G	B	—	AG	AG	AG	AG	+	—	—
sporogenes	O, S	+	D, B	B	+	AG	—	—	—	—	—	—
tetani	R, T	+	—	—	+	—	—	—	—	—	±	+

O = oval S = subterminal R = round T = terminal
A = acid G = gas C = coagulation D = digestion
B = blackening

*Litmus milk in deep tubes containing a 50 by 7 mm. piece of No. 26 gauge black stove-pipe iron.

Culture for C. diphtheriae.

Rub a sterile swab over the lesion (throat or nasopharynx).

Streak the swab over Loeffler's blood serum and incubate for 12 to 18 hours at 37°C.

a. Mix the entire growth on the surface of the slant with an inoculating needle.
b. Add a loopful to a drop of water on a slide, smear, and allow to dry.
c. Fix smear and stain with methylene blue solution.

Also streak a tellurite (Difco) plate and incubate at 37°C. for 24 hours.

a. Make smears from different colonies, fix, and stain with methylene blue solution.
b. If no diphtheria organisms are found, incubate plate for another 24 hours, and re-examine.

See Table 29 for morphology of organism. Tellurite medium should always be used in examining convalescents and contacts, while Loeffler's medium may give a quicker diagnosis in acute cases.

6. A blood agar plate should also be streaked when making a culture for diphtheria in order to rule out a streptococcus infection.
7. It is sometimes very difficult to distinguish virulent diphtheria bacilli from diphtheroid organisms by morphology and a virulence test should be made with any suspicious organisms, see page 201.

C. *Culture for Streptococci.*

1. Place the swab which has been rubbed over the throat immediately in a tube containing 2 cc. of glucose broth and twirl to obtain all the material in suspension.
2. Streak a blood agar plate with one loopful of the suspension.
3. Inoculate a tube of thioglycollate glucose broth.
4. Incubate the blood plate in 10% CO_2 and the thioglycollate broth aerobically; examine in 24 hours for streptococci or predominating organisms.
5. Stained smears must be made of hemolytic colonies to be certain they are streptococci.

TABLE 31. DIFFERENTIATION OF STREPTOCOCCI (SHERMAN, BACT. REV.)

Division	Group or Species	Lancefield Group	Hemolysis	Growth at		Hydrolyzed		Esculin Split	Active Fibrinolysis	Gelatin Liquified	Milk Curdled	Final pH in Glucose Broth	Acid produced from:										
				10° C.	45° C.	Na hippurate	Starch						Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin
Pyogenic	S. pyogenes	A	+	−	−	−	−	±	+	−	−	6.0-4.8	−	+	+	+	+	−	−	−	±	−	±
	S. mastitidis	B	+*	−	−	+	−	−	−	−	+	4.8-4.2	−	+	+	+	+	−	−	−	−	−	±
	S. equii	C	+	−	−	−			−	−	−	5.5-4.8	−	+	+	−	−	−	−	−	−	−	+
	"Animal pyogenes"	C	+	−	−	−	+	+	−	−	−	5.0-4.6	−	+	+	+	−	−	−	−	−	+	+
	"Human C"	C	+	−	−	−	±	±	±	−	±	5.4-4.6	−	+	+	±	+	−	−	+	−	−	±
	"Minute Hemolytic"	F	+	−	−	−	−	+	−	−	−	5.4-4.6	−	+	+	±	±	−	−	−	−	−	±
	Group G Streptococci	G	+	−	−	−	±	±	±	−	±	6.0-4.6	−	+	+	±	+	±	−	±	−	−	+
	Group E Streptococci	E	+	−	−	−	±	±	−	−	−	4.8-4.2	−	+	±	+	+	−	−	±	±	+	±
	Group H Streptococci	H	±	−	+	−	−	+	−	−	−	5.0-4.5	−	+	+	±	±	+	−	−	−	−	+
Viridans	S. salivarius		−	−	±	−	−	±		−	±	5.4-4.0	−	+	+	+	±	±	±	−	−	−	±
	S. equinus		−	−	+	−	±	+		−	−	4.5-4.0	−	+	±	−	±	±	±	−	−	−	±
	S. bovis		−	−	+	−	+	+		−	±	4.5-4.0	±	+	+	+	±	+	±	−	±	±	+
	Other S. bovis		−	−	+	−	−	+		−	±	4.5-4.0	−	+	+	+	±	+	±	−	±	−	+
	S. thermophilus		−	−	+	−	−	−		−	+	4.5-4.0	−	+	+	+	−	±	−	−	−	−	−
Lactic	S. lactis		−	+	−	±	−	+		−	+	4.5-4.0	±	+	±	+		−	−	−	±	−	±
	S. cremoris		−	+	−	−	−	±		−	+	4.6-4.0	−	±	±	+		−	−	−	−	−	±
Enterococcus	S. fecalis	D	−	+	+	±	−	+	−	−	+	4.5-4.0	±	+	+	+	+	±	−	±	+	+	+
	S. liquefaciens	D	−	+	+	±	−	+	−	+	+	4.5-4.0	±	+	+	+	+	±	−	+	+	+	+
	S. zymogenes	D	+	+	+	±	−	+	−	±	+	4.5-4.0	±	+	+	+	+	±	−	+	+	+	+
	S. durans	D	+	+	+	±	−	+	−	−	+	4.5-4.0	−	+	+	+	±	−	−	−	−	−	±

*Double zone of hemolysis.

eincubate for 24 hours and examine for minte beta hemolytic streptococci (Lancefield roup F).

efrigerate for 24 hours and examine for doule zones of hemolysis produced by Lanceeld's group B.

or differentiation see Table 31.

'ulture for H. pertussis.

Petri dishes containing glycerin-potato agar with 50% blood (Bordet-Gengou medium) are necessary.

TABLE 32. DISTINGUISHING CHARACTERISTICS OF H. PERTUSSIS, B. PARAPERTUSSIS, AND BR. BRONCHISEPTICA

Characteristic	H. pertussis	B. parapertussis	Br. bronchiseptica
th on agar without blood	−	+	+
n pigment produced in medium	−	+	−
lity	−	−	+
ction of nitrates to nitrites	−	−	±

Hold an uncovered plate 4 or 5 inches in front of the patient's mouth during several expulsive coughs. Cover plate as soon as possible. More positive plates will be obtained if 4 or 5 drops of penicillin solution (1000 units per cc.) are spread over one half of the plate just before exposure; the other half of the plate is kept as a control.

If unable to get patient to cough, one may streak a plate with thick, tenacious strands of fresh sputum after they are washed in three changes of sterile 0.85% NaCl solution.

The best material for culture is obtained from the nasopharnyx; if in the process of passing a nasopharyngeal swab a cough is induced, the chances of a positive culture are increased.

a. Place a loopful of penicillin solution (1000 units per cc.) on the medium at the edge of the plate.
b. Pass the swab back and forth several times through the drop of penicillin and then streak the plate with an inoculating loop.

Incubate in a 37°C. incubator for 4 to 5 days.

Examine the plate several times during the first 48 hours to detect molds and other spreading colonies which might overgrow the plate.

Remove the agar supporting such spreaders with a sterile needle or scalpel.

After 48 hours examine plates twice daily for colonies of H. pertussis.

a. Colonies are not over 1 mm. in diameter.
b. They are smooth, raised, glistening, pearly, almost transparent.
c. The area of hemolysis is indistinct.

9. H. pertussis is a gram-negative, coccoid bacillus (less than 1 micron in length), stains faintly, occurs singly and in pairs, occasionally in short chains.
10. *Slide Agglutination Test.*
    a. Suspend the suspected colonies in a drop of 0.85% NaCl solution on one end of the slide.
    b. On the opposite end of the slide, mix several loopfuls of this suspension with several loopfuls of H. pertussis antiserum diluted one tenth the titer of the serum.
    c. If the organism is H. pertussis there will be almost immediate agglutination with the serum, while the control without serum remains uniform with no clumping.
11. The Bacillus parapertussis and Brucella bronchiseptica are sometimes encountered; they are morphologically indistinguishable from H. pertussis and have similar colonies except they develop more rapidly and grow to a larger size. Cross-agglutination may occur in a relatively high titer. For differentiation of these organisms, see Table 32.

VII. Sputum Culture.

A. *Collection of Sputum.*

1. Instruct patient to wash mouth with an antiseptic.
2. Have him cough up sputum from the lungs into a sterile Petri dish and bring at once to the laboratory.

B. *Isolating Organisms.*

1. Make 2 smears; stain one by Gram's method and the other by Ziehl-Neelsen method for tubercle bacilli.
2. Examine and record the organisms found.
3. Add an equal volume of sterile 0.85% NaCl solution to the sputum.
4. Wash it by drawing it back and forth in the saline by means of a sterile 10 cc. syringe without a needle.

5. Inoculate a loopful of suspended sputum into each of the following media:
    a. Streak two blood agar plates; incubate one aerobically and one anaerobically.
    b. Inoculate a tube of glucose agar, tryptose broth, and thioglycollate glucose broth; incubate aerobically.
6. After 24 hours incubation all blood agar plates and the glucose agar slant are examined for different types of colonies.
    a. Great care must be taken to pick all types of colonies for smears and subcultures.
    b. If pure cultures are needed for further identification or for a vaccine, streak typical colonies of the various organisms on blood agar plates.
7. Make smears of all broth cultures and also streak a loopful of the broth cultures on blood agar plates for further identification of the organisms.
8. All subcultures are incubated as the original cultures and one subculture of the thioglycollate glucose broth is incubated anaerobically.
9. When all the aerobic and anaerobic organisms have been identified and obtained in pure culture, save until certain that a vaccine is not wanted.
10. If a vaccine is ordered, pick typical smooth colonies, one for each type of organism, and subculture on suitable media for aerobes and anaerobes.
11. Make a vaccine from each organism as described under autogenous vaccine (p. 197).
12. *Organisms Most Likely to be Isolated.*
    Streptococci
    Diplococcus pneumoniae
    Hemophilus hemolyticus
    Staphylococci
    Neisseria catarrhalis
    Diphtheroids
    Hemophilus influenzae
    Klebsiella pneumoniae

VIII. Blood Culture.

A. *General Considerations.*

1. The bacteriologist must be informed of the following when a blood culture is ordered.
    a. The nature of the suspected infection so that proper media can be used.
    b. The time when the patient's temperature is rising so that the blood can be drawn at that time.
    c. If the patient is receiving sulfonamide therapy, the culture media used must contain 5 mg. of para-aminobenzoic acid per 100 cc.
    d. If the patient is receiving penicillin therapy, penicillinase must be added to the media; see page 166.
2. Blood should not be drawn for a blood culture within 8 hours after an intravenous injection of any kind; if the injection was followed by a chill or thermal reaction, it should not be drawn within 20 hours after the injection.
3. Blood culture tray should include the following:
    a. Tincture of iodine.
    b. Tincture of green soap.
    c. Alcohol—70%.
    d. Sterile gauze sponges.
    e. Two sterile swabs.
    f. Sterile towel.
    g. Sterile 20 cc. syringe.
    h. Sterile 21 gauge needle.
    i. Culture media.
    j. Sterile Petri dishes (3).
    k. Alcohol lamp.
    l. Matches.
4. It is preferable to add the blood directly to the media at the bedside, but it may be placed in a small sterile flask containing 5 cc. of a 2% sodium citrate solution and the cultures made in the laboratory.
5. The mouths of all flasks must be flamed after removing cotton plugs and before replacing them.
6. Blood cultures should be incubated until growth appears or for 4 weeks.
7. *Organisms Most Likely to be Isolated.*
    Diplococcus pneumoniae
    Streptococci
    Staphylococci
    Salmonella typhosa
    Coliform group
    Neisseria gonorrhoeae
    Neisseria meningitidis
    Brucella group
    Pasteurella tularensis

B. *Routine.*

1. Close all windows and transoms in patient's room before preparing patient's arm.
2. Look at both arms to see which has the best vein for bleeding.
3. Place sterile towel on bed under forearm and elbow.
4. Prepare the arm at the elbow as follows:
    a. Wash with tincture of green soap.
    b. Remove the soap with a sterile sponge.
    c. Apply tincture of iodine over a prominent vein with a sterile swab and leave for 1 minute before removing.
    d. With another sterile swab remove the

excess of iodine with 70% alcohol, applying the swab first to the area to be punctured and not touching the area again.

With aseptic precautions withdraw 15 cc. of blood from the vein in a sterile 20 cc. syringe.

The following procedures must be carried out quickly at the bedside before the blood coagulates and with strict asepsis.

a. Place 5 cc. of blood in one flask containing 100 cc. of plain broth, 5 cc. in a flask containing 100 cc. of tryptose broth, and 2 cc. in a tube containing 25 cc. of thioglycollate glucose broth, all of which have been warmed to body temperature.

b. Place 3 cc. of blood in a flask containing 50 cc. of plain agar to which 10 cc. of plain broth had been added before it was melted and cooled to 45°C.

c. Make 3 blood agar plates by pouring this mixture into 3 sterile Petri dishes.

Incubate 2 blood plates and the tryptose broth in 10% CO_2 and all others under aerobic conditions.

If growth appears, make smears and stain by Gram's method.

If there is no cloudiness in the broths in 3 days, shake well and subculture on blood agar plates as follows:

a. One plate from tryptose broth and incubate in 10% CO_2.

b. One plate from plain broth and incubate aerobically.

c. Two plates from thioglycollate glucose broth, incubate one aerobically and one anaerobically.

Make new subcultures of the broths at the end of the first week and then at weekly intervals for 3 more weeks, unless growth is obtained earlier.

Subculture surface colonies from blood agar plates into broth. This broth culture (if pure) may be used for the inoculation of sugar media for further identification.

If deep colonies appear, cut them out, place in a tube of broth, and break up the colony with an inoculating loop.

Incubate broth cultures for 4 weeks and blood plates for 2 weeks if no growth appears.

For D. pneumoniae.

Use the same method as for routine blood culture except use glucose broth instead of plain broth.

Watch carefully for growth from 12 to 24 hours after taking the culture as pneumococci autolyze quickly and are easily lost.

3. Subculture the inoculated glucose broth routinely on a blood agar plate after 18 hours.

D. *For N. gonorrhoeae or meningitidis.*

1. Use same method as for routine blood culture except use glucose infusion agar and glucose infusion broth instead of plain agar and plain broth.
2. The media should be inoculated at the bedside.
3. Incubate all cultures in 10% CO_2 and examine after 2 days.
4. If there is no growth, reincubate for an additional 7 days, examining every 2 days.
5. Examination of cultures should consist of making gram-stained smears and subculturing one loopful of the well shaken broth cultures onto freshly prepared chocolate agar plates which are incubated in 10% CO_2 for 48 hours.

E. *For Brucella.*

1. Obtain blood as for a routine blood culture and place 5 cc. in 75 cc. of trypticase soy broth which has had 4 cc. of a sterile 20% sodium citrate solution added to it. Incubate in 10% CO_2.
2. Subculture 0.5 cc. of the above broth well shaken onto a trypticase soy agar plate after 4 days and continue to subculture every 2 days for 1 month.
3. Castaneda's medium is excellent for routine use in blood cultures to detect Brucella.
   a. Formula:

Trypticase soy agar	2.0 gm.
Sodium chloride	0.5 gm.
Sodium citrate	0.5 gm.
Agar	3.0 gm.
Distilled water	100.0 cc.

   b. Place 15 cc. in 100 cc., flat-sided, rectangular bottles or in 4 oz. prescription bottles that have one flat side.
   c. Plug the mouth of the bottle (about 1¼ cm. in diameter) with cotton, cover with thick paper, and sterilize at 15 lbs. pressure for 15 minutes.
   d. Place the bottles on their flat side for several days so that the agar sets on the wall forming a hard, even, transparent layer.
   e. Add 10 cc. of broth with aseptic precautions. This broth is sterilized separately at 15 lbs. pressure for 15 minutes and contains 2% trypticase soy broth and 2% sodium citrate.
   f. Close the bottles with sterile rubber vaccine stoppers, inject enough CO_2 to give a 10% mixture, and again cover with the same paper cap.
   g. Test for sterility by placing in the 37°C.

incubator in a vertical position for 3 or 4 days, wetting the agar surface with broth at 24 hour intervals.

- h. Inoculate with 10 cc. of blood, allow the mixture of blood and broth to spread over the agar for a moment, and then incubate in a vertical position at 37°C.
- i. Every other day tip the bottle so that the broth flows over the agar layer, then replace in the incubator in a vertical position.
- j. *Results:*
  - 1) Colonies appearing on the agar from 24 to 48 hours are apt to be due to contamination.
  - 2) Colonies appearing 24 to 48 hours after the second inoculation are usually Salmonella, Staphylococcus, or Streptococcus, but may be Brucella.
  - 3) Positive cultures of Brucella most frequently appear between the 6th and 10th day of cultivation.
  - 4) The culture should be kept 20 days before discarding.

F. *For P. tularensis.*

1. Obtain 10 cc. of blood (not citrated) as described under routine blood culture.
2. Place 0.5 to 1 cc. on each of 6 or 7 glucose-cystine blood agar slants and one plain infusion agar slant which serves as a control. The cystine blood agar slants should be previously placed in the 37°C. incubator for 24 hours to evaporate any water of condensation.
3. Incubate aerobically.
4. Growth will appear only on the cystine media if it is P. tularensis.
5. Incubate for 3 weeks before reporting negative.

G. *For Cl. perfringens* (Gas Bacillus).

1. Inoculate one or more loopfuls of blood from the heart at autopsy into tubes of milk which have been kept in boiling water for 30 minutes and cooled to room temperature.
2. Incubate at 37°C. in an anaerobe jar for 24 to 48 hours.
3. Do not heat these inoculated tubes at 80°C. as in a culture of feces because the organisms exist only in the vegetative state in the blood.

IX. Urine Culture.

A. *General Considerations.*

1. The urine must be a catheterized specimen and kept free from contamination.
2. Centrifuge entire specimen in 50 cc. centrifuge tubes at a high rate of speed for 30 minutes and pour off the supernatant urine.
3. If a culture for tubercle bacilli is wanted, centrifuge for 45 minutes and see directions for that culture.
4. Make cultures as described below and then make 2 smears of the sediment.
   - a. Place a cover glass on one smear and examine while wet. Report findings as in a routine urine examination (see Table 1, p. 3).
   - b. Let the other smear dry and stain for tubercle bacilli.

B. *Routine Culture.*

1. Inoculate a glucose agar slant and a tube of thioglycollate glucose broth each with one loopful of the sediment.
2. Streak a blood agar plate and an E.M.B. plate each with one loopful of the sediment.
3. Incubate all cultures for 24 hours or longer if no growth appears.
4. Make smears of the broth culture and agar slant if there is growth. Make subcultures on 2 blood agar plates from the thioglycollate broth; incubate one aerobically and one anaerobically.
5. Pick colonies from the plates, make smears, and stain by Gram's method.
6. Subculture colonies for further identification.
7. *Organisms Most Likely to be Isolated.*
   Escherichia coli
   Staphylococci
   Streptococci
   Proteus
   Aerobacter aerogenes
   Diphtheroids
   Bacillus subtilis
   Pseudomonas aeruginosa
   Shigella group
   Salmonella—usually enteritidis or typhosa
   Paracolobactrum group

C. *Culture for Tubercle Bacilli.*

1. Centrifuge the entire specimen in sterile 50 cc. centrifuge tubes at a high rate of speed for 45 minutes. (If specimen is small and appears concentrated, dilute with sterile distilled water to a pale straw color to lower the specific gravity of the urine.)
2. Remove the upper 1 cc. layer with a sterile pipette, decant the remainder of the supernatant fluid, and add the urine in the pipette to the sediment.
3. Make a smear of the sediment and stain with Loeffler's methylene blue solution to see if any other bacteria are present.
4. If no organisms are found, proceed as follows:
   - a. Make a smear and stain for tubercle bacilli.
   - b. Inoculate each of 3 tubes of special medium (Saenz's modification of Petragnini's

medium) with 0.25 cc. of sediment.

Incubate at 37°C. with the tubes lying almost flat to keep the medium moist.

Examine once a week for growth; incubate for 3 months if no growth appears earlier.

organisms are present in the smear stained ith methylene blue, one must use the follow- g method:

Add 2 cc. of a 5% oxalic acid solution and mix.

. Incubate at 37°C. for 30 minutes.

. Fill the tube with sterile 0.85% NaCl solution and centrifuge at a high rate of speed for 30 minutes.

. Pour off the supernatant fluid and proceed as above.

. Colonies of the tubercle bacilli appear as a cream-colored, dry, granular growth. In order to know if the organism is a pathogenic acid-fast bacilli it must be injected into a guinea pig.

Culture Medium for Tubercle Bacilli (Saenz's odification of Petragnini's).

a. Place 188 gm. of fresh milk in a 300 cc. Florence flask.

b. In a liter Erlenmeyer flask containing 75 gm. of glass balls, place 12 gm. of potato flour and 1.3 gm. of asparagine.

c. Autoclave both flasks and contents at 15 lbs. pressure for 20 minutes.

d. Let milk cool and then add to the contents in the Erlenmeyer flask with continual shaking.

e. Place in a cold water bath; heat to boiling, shaking the flask every few minutes to avoid clumping. Heat until mixture has the consistency of puree.

f. Cool to 60°C. and add 6 whole eggs and 2 yolks aseptically.
   1) Wash eggs with soap and water, dry, and burn with alcohol.
   2) Flame one end of the egg, crack the shell with sterile forceps, and pour the contents into the flask.

g. Agitate vigorously and then filter through sterile gauze into a sterile liter flask.

h. Add 15 cc. of sterile glycerin and 12 cc. of 2% malachite green.

i. Shake thoroughly and distribute 6 cc. into sterile, chemically clean tubes with bakelite screw caps.

j. Screw the caps on loosely and lay tubes at the appropriate slant in a container which has a tight lid and a layer of sand in the bottom.

k. Cover tubes with sand and place the lid of the container on tight.

l. Place in the autoclave and close both the door and the air outlet valve.

m. Turn the steam on and allow the pressure to reach 15 lbs. very slowly to avoid bubbles in the medium.

n. Maintain this pressure for 10 minutes letting no air or steam escape.

o. Open the air outlet valve so slightly that the pressure will not vary more than one-half pound thus allowing the condensed water and some of the air to escape.

p. Close the valve and sterilize at 15 lbs. pressure for 20 minutes.

q. Tighten the caps on the tubes before storing the medium in the refrigerator.

X. Feces Culture.

A. *Organisms Most Likely to be Isolated.*

Coliform group
Salmonella group
Shigella group
Pseudomonas aeruginosa
Proteus
Alpha Streptococcus fecalis
Clostridium perfringens

B. *Culture for Shigella Group (Dysenteriae) and Salmonella Group (Typhosa and Paratyphi).*

1. The specimen must be collected in a clean container, or better, a sterile one, or by rectal swab and cultures made as soon as possible.
2. If any blood tinged mucus is present, wash in sterile 0.85% NaCl solution and streak directly on E.M.B. and SS plates.
3. If specimen is solid, emulsify with a small amount of sterile 0.85% NaCl solution in a test tube. It should represent a generous amount of the whole specimen and not merely a loopful.
4. With a single loopful of the emulsion, streak 2 well-hardened plates of E.M.B. medium. Do not add any more fecal emulsion to the loop or sterilize it between plates.
5. Streak an SS plate with 5 loopfuls of the emulsion.
6. Emulsify 3-5 gm. of undiluted feces in 12 cc. of peptone-tetrathionate broth to which 0.28 cc. of iodine solution has been added immediately preceding its use.
   a. The Difco broth and iodine solution are prepared as directed on the package, but do not add the iodine solution at this time.
   b. Keep the iodine solution separate and add to broth just before using.
7. One may emulsify 1 gm. of undiluted feces in a tube of Selenite-F medium (BBL) in-

stead of tetrathionate broth or along with it. The Selenite-F medium is better for the isolation of the Salmonella group, while the tetrathionate broth is better for Shigella.

8. Incubate all cultures for 18 to 24 hours at 37°C.
9. Inoculate an SS plate with several loopfuls of the tetrathionate or Selenite-F broth culture and incubate for 24 hours.
10. Examine the original E.M.B. and SS plates for suspicious colonies.
    a. On E.M.B. plates the Escherichia (coli) group produces pink colonies with a metallic sheen; Salmonella (typhosa and paratyphi), Shigella (dysenteriae), and Proteus groups produce bluish white or colorless colonies.
    b. On SS plates colonies of Shigella and Salmonella are translucent and usually colorless or a delicate pink, while those of the Escherichia group are opaque, pink to deep red, or nearly colorless with a pink center.
11. Fish 5 to 10 suspicious colonies and subculture on an E.M.B. plate. Great care must be exercised in picking colonies, because, although the coliform bacteria on the SS plate are inhibited, they are not necessarily dead.
12. Transfer any nonlactose fermenting bacteria to Triple Sugar Iron (Difco or BBL) slants and incubate overnight at 37°C. Care must be taken to pick a pure colony.
    a. Red slant and yellow butt with or without gas indicates fermentation of glucose.
    b. A yellow slant and butt with or without gas indicates fermentation of lactose or sucrose.
    c. No change indicates neither glucose, lactose, nor sucrose attacked.
    d. Blackening of the medium reveals the production of hydrogen sulfide.
    e. Discard any slants having acid and gas throughout.
    f. Organisms from slants having a typical acid butt, with or without gas, and alkaline slant are transferred to urease test medium (BBL) for the identification of Proteus.
        1) Incubate 2 to 4 hours at 37°C.; if there is hydrolysis of the urea, Proteus is present and the culture can be discarded.
        2) If negative, incubate longer observing at 24 and 48 hours; some of the paracolon bacilli give a weak delayed reaction at 48 hours.
13. Identify any nonlactose fermenting bacteria by cultural reactions. See Table 33.
14. Final identification should be done by glutination tests with specific serum.

C. *Culture for Streptococci.*

1. Place a loopful of feces in 5 cc. of a ste 1% solution of sodium carbonate and inc bate 2 hours.
2. Make 3 dilutions of this sodium carbonat culture in the following manner:
    a. Place 5 cc. of sterile 0.85% NaCl solutio in each of 3 sterile test tubes marked 2, and 3.
    b. In tube 1 place 0.1 cc. of the sodium car bonate culture, making a 1-50 dilution.
    c. From tube 1 transfer 0.1 cc. to tube making a 1-2500 dilution.
    d. From tube 2 transfer 0.1 cc. to tube making a 1-125,000 dilution.
    e. Again label 3 sterile test tubes, 1, 2, and Place 0.1 cc. of each of the above dilu tions in the corresponding test tube.
3. Pour approximately 10 cc. of melted aga which has been cooled to 45°C. into eac of the 3 tubes and add 0.5 cc. of sterile de fibrinated blood to each.
4. Mix thoroughly and pour the contents o each tube into a Petri dish.
5. Incubate 24 hours or longer if necessary.
6. Report number of bacteria in 1 cc. of fece by counting the colonies on the plate havin the smallest number of colonies, multiply b the dilution of that plate, then multiply by which represents the dilution in the sodiu carbonate.
7. Cut out one or more colonies with a needl and place in tubes of glucose broth for furthe identification.

D. *Culture for Cl. perfringens (Gas Bacillus)*

1. Emulsify a large loopful of feces in 5 cc. o sterile 0.85% NaCl solution.
2. Heat 3 tubes of sterile milk in boiling wate for 30 minutes to drive off all the air in th milk.
3. Cool to 80°C. and mark tubes 1, 2, and
4. Inoculate No. 1 with 0.1 cc. of fecal emulsion No. 2 with 0.2 cc., and No. 3 with 0.3 cc
5. Keep at 80°C. for 20 minutes and then in cubate at 37°C.
6. Examine tubes after 4 to 12 hours.
7. A positive culture for the gas bacillus wil have the following characteristics:
    a. The casein is mostly dissolved (80%).
    b. The residual casein is filled with holes du to gas ("stormy fermentation").
    c. The culture has an odor of rancid butter due to the formation of butyric acid.
8. The gas bacillus is found in both vegetativ

	Motili	Butt	Slant	H_2S	Gluco	Lacto	Sucros	Malto	Salici	Xylos	Dulci	Mann	Rhan	Citra	Gelat Lique	Indol	Litm Milk
Escherichia Group																	
E. coli (communis)	±	AG	A	—	AG	AG	—	AG	AG	AG	V	AG	AG	—	—	+	A, Coag.
E. coli communior	±	AG	A	—	AG	AG	AG	AG	—	AG	V	AG	AG	—	—	+	A, Coag.
E. coli freundii	±	AG	A	+	AG	AG	V	AG	V	AG	V	AG	AG	+	V	V	A, Coag. (V)
E. coli intermedium	±	AG	A	—	AG	AG	V	AG	V	AG	V	AG	AG	+	—	V	A, Coag. (V)
Aerobacter Group																	
A. aerogenes	±	AG	A	—	AG	AG	V	AG	AG	AG	V	AG	AG	+	—	V	A, Coag.
A. cloacae	+	AG	A	—	AG	AG	AG	AG	AG	AG	—	AG	—	+	+	—	A, Coag. Pep. ±
Paracolobactrum Group																	
P. aerogenoides	±	AG	A-Alk	—	AG	AG±	V	AG	AG	AG	V	AG	V	+	V	V	A, Coag.
P. intermedium	±	AG	A-Alk	V	AG	AG±	V	AG	V	AG	V	AG	AG	+	V	V	A, Coag.
P. coliforme	±	AG	A-Alk	—	AG	AG±	V	AG	V	AG	V	AG	AG	—	—	+	A, Coag.
Alcaligenes fecalis	+	—	Alk	+	—	—	—	—	—	—	—	—	—	—	—	—	Alk.
K. pneumoniae (B. friedländeri)	—	AG	A	—	AG	AG	AG	AG	AG	AG	—	AG	AG	V	—	—	A(V)
Ps. aeruginosa (B. pyocyaneus)	+	A	Alk	—	A	—	—	—	—	—	—	—	—	+	+	—	Alk., Coag. Pep.
Proteus Group																	
Pr. vulgaris	+	AG	A	+	AG	—	AG	AG	V	AG	—	—	—	+	+	+	A-Alk. Pep.
Pr. mirabilis	+	AG	A-Alk	+	AG	—	AG±	—	V	AG	—	—	—	+	+	—	A-Alk. Pep.
Pr. morganii	+	AG	Alk	—	AG	—	—	—	—	—	—	—	—	—	—	+	Neut-Alk.
Pr. rettgeri	—	A	A-Alk	—	A	—	A±	—	A	A	—	A	—	+	—	+	Neut-Alk.
Salmonella Group																	
S. typhosa	+	A	Alk	+	A	—	—	A	—	A	—	A	—	V	—	—	A-Alk.
S. paratyphi	+	AG	Alk	—	AG	—	—	AG	—	—	AG	AG	AG	—	—	—	Sl. A.
S. schottmulleri (1) (paratyphi B)	+	AG	Alk	+	AG	—	—	AG	—	AG	AG	AG	AG	V	—	—	A-Alk.
S. hirschfeldii	+	AG	Alk	+	AG	—	—	AG	—	AG	AG	AG	AG	+	—	—	A-Alk.
S. choleraesuis (suipestifer)	+	AG	Alk	—	AG	—	—	AG	—	AG	AG	AG	AG	+	—	—	A-Alk
S. abortivoequina	+	AG	Alk	—	AG	—	—	AG	—	AG	AG	AG	AG	+	—	—	A-Alk.
S. pullorum	—	AG	Alk	+	AG	—	—	—	—	AG±	—	AG	AG	—	—	—	A-Alk.
S. gallinarum	—	A	Alk	V	A	—	—	A	—	A	A	A	A	—	—	—	Neg.
Shigella Group																	
S. dysenteriae (Shiga)	—	A	Alk	—	A	—	—	—	—	—	—	—	—	—	—	—	A-Alk.
S. paradysenteriae (Flexner)	—	A	Alk	—	A	—	—	—	—	—	—	A	A	—	—	+	A-Alk.
S. ambigua (Schmitz)	—	A	Alk	—	A	—	—	—	—	—	—	—	A	—	—	+	A-Alk.
S. sonnei	—	A	A-Alk	—	A	A±	A±	A±	—	—	—	A	A±	—	—	—	A, Coag.
S. alkalescens	—	A	Alk	—	A	—	V	A	—	A	A	A	A	—	—	+	Alk.
S. sp (newcastle)	—	AG	Alk	—	AG	—	—	AG±	—	AG	AG±	—	—	—	—	—	A-Alk.
S. ceylonensis (dispar)	—	A	A	—	A	A±	A	A	—	A	A	A	A	—	—	+	A, Coag.

(1) S. typhimurium (aertryche) and S. enteritidis have the same reactions as S. schottmülleri.
A = Acid G = Gas V = Variable ± = weak or delayed reaction.

and spore forms in the feces. Because there are so many other bacteria present, the cultures are heated after inoculation to kill these organisms. The spores of the gas bacilli are not killed and reproduce in the milk.

XI. Urethral and Vaginal Cultures for N. Gonorrhoeae.

A. *Collection of Material.*

1. Collect the material on swabs.
2. If the swabs cannot be sent to the laboratory at once, place in 1 cc. of broth (proteose peptone No. 3) and keep in the refrigerator until sent to the laboratory.
    a. *Preparation of swabs.*
        1) Make small cotton swabs by dipping the end of an applicator stick into a warmed gelatin mixture and then wrapping cotton tightly around the tip.
        2) Place in test tubes and sterilize in the autoclave.
        3) *Gelatin mixture.*

Bacto-gelatin	4.0 gm.
Na_2HPO_4	0.2 gm.
Distilled water	40 cc.

Heat in a boiling water bath until gelatin is dissolved and then keep in the refrigerator.

    b. *Preparation of broth.*

Proteose peptone No. 3	2.0 gm.
NaCl	0.5 gm.
Soluble starch	1.0 gm.
Distilled water	100 cc.

        1) Heat in a boiling water bath until peptone is dissolved and then filter.
        2) Distribute 1 cc. in test tubes and autoclave at 15 lbs. pressure for 20 minutes.

B. *Cultures.*

1. Prepare plates of "glucose chocolate agar" (so-called because of its color, not because it contains chocolate).
    a. *Bacto GC Medium Base (Difco).*
        1) Place 7.2 gm. of the agar in a 250 cc. Erlenmeyer flask and add in small portions 100 cc. of distilled water, mixing with a stirring rod between each addition.
        2) Place in a pan of water and heat until the water boils (not the agar).
        3) Distribute 10 cc. in culture tubes and autoclave at 15 lbs. pressure for 20 minutes.
    b. *Bacto-Hemoglobin (Difco).*
        1) Dissolve 2 gm. of the hemoglobin in 100 cc. of distilled water in the same manner as the agar but do not heat in a water bath.
        2) When solution is nearly complete, filter through coarse, moistened gauze to remove undissolved particles.
        3) Distribute 10 cc. in culture tubes and autoclave at 15 lbs. for 20 minutes.
    c. The above 2 preparations keep indefinitely in the refrigerator.
    d. When plates are needed, melt 10 cc. of the proteose agar for each plate in boiling water and cool to 60°C. Add 0.2 cc. of Bacto-Supplement A or Bacto-Supplement B.
    e. Warm the hemoglobin solution to 60°C.
    f. Mix 10 cc. of the hemoglobin solution, under aseptic conditions, with 10 cc. of agar and pour into a sterile Petri dish.
    g. Incubate overnight for sterility.
    h. Plates can be kept for only 4 days before using.
2. Touch the surface of the media close to the outer edge of the plate lightly with the swab.
    a. If broth is used, remove the swab and centrifuge the broth.
    b. Pour off the supernatant fluid and inoculate the plate with a loopful of the sediment.
3. With a wire loop spread this inoculum by streaking the plate.
4. Place moist cotton in the bottom of the CO_2 jar and place inverted plates in the jar.
5. Examine after incubating 24 hours. If no growth is found, replace plates in CO_2 jar and incubate again.
6. Colonies of gonococci are convex, transparent, from 1 to 3 mm. in diameter, with undulated margins, and contain oxydase.
7. Add a drop of a 1% aqueous solution of para-aminodimethylaniline monohydrochloride to suspicious colonies (make dye fresh every 2 or 3 days and keep in the refrigerator).
8. Oxydase positive colonies turn first pink, then maroon, and finally black.
9. Subcultures should be made before the indicator is added to the colonies or while the colonies are still pink after adding the dye. The organisms are dead when the colony turns black.
10. All of the Neisseria group are oxydase positive as well as the Hemophilus group, certain yeasts, B. subtilis, streptothrix, and certain varieties of coliform bacilli.
11. To identify gonococci inoculate semisolid sugar media. See Table 34.

TABLE 34. DIFFERENTIATION OF NEISSERIA*

Species	Glucose	Maltose	Levulose	Sucrose	Growth on plain agar	Growth at 22° C.	Description of Colony
rrhoeae	A**	–	–	–	–	–	Small, round, convex.
ingitidis	A	A	–	–	–	–	Small, round, bluish gray.
rrhalis	–	–	–	–	+	+	Large, grayish white.
a	A	A	A	A	+	+	Large, wrinkled, impossible to emulsify.
lava	A	A	A	A	+	+	Greenish yellow, adherent to medium.
a	A	A	A	–	–	–	Yellow.
lava	A	A	–	–	±	±	Greenish yellow, adherent to medium.
escens	–	–	–	–	?	?	Golden yellow.

phology—Gram-negative diplococci.
dicates acid formation.

XII. Cerebrospinal Fluid Culture.

irect Smears.

rect smears are very important because the
pe of meningitis may often be diagnosed
om them.

entrifuge the specimen at high speed for 15
inutes and then pour the supernatant fluid
to another tube for other tests keeping the
diment free from contamination.

ake cultures as described below and 2
nears of the sediment.

ain one smear by Gram's method and ex-
nine especially for the following organisms.

eisseria meningitidis
reptococci
iplococcus pneumoniae
aphylococci
emophilus influenzae

ain the other smear by the Ziehl-Neelsen
ethod and examine for M. tuberculosis.

neumococci and H. influenzae (Group B)
ay be typed directly from the spinal fluid
y the Quellung reaction, see page 192.

ultures.

is very important that the spinal fluid be
ept at body temperature and cultures made
soon as possible. If cultures can not be
ade immediately, place the fluid in the 37°C.
icubator until they can be made; spinal fluid
self is a good culture medium.

possible, take 2 blood agar slants warmed
body temperature to the patient's bed-
de when the spinal puncture is being made.

. Inoculate each slant with 1 to 2 cc. of
fluid directly from the needle.

. Take to the laboratory at once and in-
cubate under 10% CO_2 tension.

noculate a loopful of sediment (see No. 2
nder direct smear above) to each of the
ollowing tubes and plates: 2 blood agar
lates, 2 tubes of blood infusion broth, 2
tubes of glucose broth, also 1 tube of thioglycollate glucose broth.

4. Incubate 1 blood agar plate, 1 tube of blood infusion broth, and 1 tube of glucose broth in 10% CO_2; incubate the other cultures aerobically.
5. For gonococcus cultures in suspected gonococcal meningitis, see urethral and vaginal cultures for N. gonorrhoeae.
6. For tubercle bacilli inoculate several tubes of Saenz's medium (see p. 179); a guinea pig should also be inoculated with a portion of the sediment.

XIII. Culture of Fluids (Joint, Pleural, Peritoneal, etc).

A. *Transudates.*

1. Centrifuge the fluid at high speed for 30 minutes and discard the supernatant fluid.
2. Inoculate each of the following with 1 loopful of sediment: 2 blood agar plates, 1 glucose agar slant, 1 tube of glucose broth, and 1 tube of thioglycollate glucose broth.
3. Incubate one blood agar plate in 10% CO_2 and the other cultures aerobically.
4. Peritoneal fluid should also be cultured as follows:
   a. Streak an E.M.B. plate with a loopful of sediment.
   b. Inoculate 1 cc. of sterile 1% sodium carbonate solution with several loopfuls of sediment, incubate for 2 to 4 hours at 37°C., and then streak a loopful on a blood agar plate.

B. *Joint (Synovial) Fluid.*

1. If the fluid does not coagulate, follow directions under transudates (A) and, in addition, streak a chocolate agar plate for N. gonorrhoeae as described under urethral and vaginal cultures.
2. If the fluid coagulates, obtain as much fluid as possible with a sterile pipette and inoculate the following:

   a. One tube of thioglycollate glucose broth; if growth occurs in 48 hours subculture on 2 blood agar plates, incubate 1 aerobically and 1 anaerobically.
   b. A chocolate agar plate for N. gonorrhoeae and incubate in 10% CO_2.
3. Incubate the remaining coagulum in 10% CO_2.

XIV. Culture of Tissues (Tonsils, etc.).

A. *Preparation of Tissue.*

1. Remove the tissue from the specimen bottle with sterile forceps and place in a small sterile mortar containing a very small amount of sterile sand.
2. Tonsils should be immersed in boiling water for 10 seconds before placing in the mortar.
3. Grind the tissue thoroughly using a sterile pestle and adding sterile 0.85% NaCl solution in very small portions until 3 or 4 cc. have been added.
4. This suspension may be used for animal inoculation.

B. *Culture.*

1. Streak each of 2 blood agar plates and 1 glucose agar slant with a loopful of the suspension.
2. With a sterile pipette transfer 0.2 to 0.3 cc. of the suspension to a tube of glucose broth and a tube of thioglycollate glucose broth.
3. Incubate all cultures aerobically except one blood agar plate which should be incubated in 10% CO_2.

XV. Culture of Extracted Teeth.

A. *Preparation of Tooth.*

1. Drop tooth into 95% alcohol, remove immediately with sterile forceps, and pass through a flame to burn off the alcohol.
2. Repeat this process twice and then place the tooth in a sterile Petri dish.

B. *Culture.*

1. Hold the tooth with sterile forceps and rub the outside of it gently over a section of a blood agar plate as a test for sterility.
2. Place the tooth in a sterile towel.
3. Clip off the apex of the tooth with a sterile bone forceps (or lineman's pliers) and drop it into a tube of thioglycollate glucose broth.
4. Hold the tooth in a sterile towel and crack it with the forceps or pliers.
5. Culture the pulp on a blood agar plate and in glucose broth and thioglycollate glucose broth; incubate aerobically.

XVI. Culture for Fungi.

See Section on Mycology, page 204.

Special Tests

I. Solubility Tests for Pneumococci.

A. *Preparation of Bile.*

1. Place 100 cc. of ox bile in a flask and
   clave at 15 lbs. pressure for 15 minute
2. Pass through filter paper until absolutely
3. Distribute in tubes, 10 cc. to a tube, and
   clave again at 15 lbs. pressure for 15 min

B. *Bile Solubility Test.*

1. Label 3 small test tubes, A, B, and C.
   a. In A place 0.5 cc. of bile and 0.5
      0.85% NaCl solution.
   b. In B place 0.5 cc. of bile and 0.5 cc.
      24 hour broth culture of the organis
      be tested.
   c. In C place 0.5 cc. of 0.85% NaCl sol
      and 0.5 cc. of the broth culture.
2. Incubate tubes at 37°C. for 2 hours.
3. *Results:*
   a. If tube A is clear and tubes B and C
      cloudy, the organism is insoluble in
   b. If tube C is cloudy and tubes A and B
      clear, the organism is soluble in bile.
   c. All types of pneumococci are bile
      ble.
   d. Most strains of streptococci are inso
      in bile.

C. *Sodium Lauryl Sulfate Solubility Test.*

1. Make a 2% aqueous solution of crude sod
   lauryl sulfate ("Dreft").
2. Heat to dissolve, filter, and keep in a
   stoppered bottle in a 37°C. incubator.
3. Add 0.1 cc. of the 2% sodium lauryl su
   solution to 0.9 cc. of a 24 hour broth
   ture of the organism to be tested.
4. For a control add 0.1 cc. of 0.85% NaC
   lution to 0.9 cc. of the broth culture.
5. Incubate both tubes for 30 minutes.
6. If the organism present is pneumococcus,
   tube containing the sodium lauryl sulfate
   lution will be clear while the control
   will remain cloudy.

II. Indole Test.

A. *Reagent.*

Para-dimethylaminobenzaldehyde	
Ethyl alcohol (95%)	9
Hydrochloric acid (conc.)	2

B. *Method.*

1. Layer 2 or 3 cc. of the reagent on the
   face of a 24-48 hour Bacto-tryptone cul
2. If indole is present, a pink color appears a
   zone of contact of the broth and reagen
3. If a pink color appears, shake the tube
   tributing the color throughout. (If the
   disappears, add more reagent.)
4. Add 1 or 2 cc. of chloroform and shake
   tube.
5. Allow the chloroform to settle; if the
   appears only in the chloroform it is a
   indole reaction.

III. Coagulase Test for Staphylococci.

Materials Required.

Plasma from citrated human blood (0.08 gm. of sodium citrate to 10 cc. of blood).

a. Blood must be obtained from a person whose plasma is known to be coagulated by a known coagulase positive staphylococcus.

b. Plasma must be fresh.

A 24 hour broth culture of staphylococcus or a 24 hour agar culture.

Method.

Add 0.5 cc. of the broth culture or a large loopful of organisms from the agar slant to 0.5 cc. of plasma.

For a control add 0.5 cc. of broth to 0.5 cc. of plasma.

Place both tubes in a 37°C. water bath and observe at 30 minutes, 1, 2, 3, and 24 hours for coagulation. The control should show no coagulation.

Interpretation.

The shorter the period required for the staphylococcus to coagulate the plasma the more strongly positive the results; if coagulation occurs within 24 hours the test is considered positive.

A positive coagulase test, especially when combined with hemolytic activity, usually indicates a pathogenic toxin-producing strain of staphylococcus.

IV. Fibrinolytic Activity of Hemolytic Streptococci.

Material Required.

Sterile Plasma from oxalated human blood (0.02 gm. of potassium oxalate to 10 cc. of blood).

   a. The plasma must be of known sensitivity.
   b. Plasma from certain individuals, notably those who have recovered recently from hemolytic streptococcus infections, are highly resistant to fibrinolysis.

2. *Sterile 0.85% NaCl Solution.*
3. *Sterile 0.25% Calcium Chloride Solution.*
4. *Streptococcus Culture*—a turbid 18-24 hour broth culture.

B. *Method.*

1. Dilute 0.2 cc. of plasma with 0.8 cc. of 0.85% NaCl solution.
2. Add 0.5 cc. of the streptococcus culture to be tested.
3. Mix immediately and add 0.25 cc. of a 0.25% calcium chloride solution.
4. Mix and place in a water bath at 37°C.
5. In about 10 minutes there should be a solid coagulum.
6. Observe frequently and note the time when the contents of the tube again become completely fluid.
7. If the clot is not dissolved in 24 hours, the streptococcus is considered negative for fibrinolytic activity.

V. Hemolysin Test for Streptococci.

A. *Materials Required.*

1. A 24 hour broth culture of the streptococcus to be tested.
2. A 5% suspension of washed rabbit erythrocytes in 0.85% NaCl solution.

B. *Test.*

1. Add 0.5 cc. of the broth culture to 0.5 cc. of the washed erythrocytes.
2. Incubate at 37°C. for 2 hours.
3. If hemolysis is not evident, centrifuge to detect a slight hemolysis.

VI. Penicillin Assay.

A. *Reagents Required.*

1. *Sterile 0.85% NaCl Solution.*
2. *Fluid* to be tested, i.e., serum, urine, spinal fluid, etc. (Fluids may be kept frozen for 24 hours without changing the penicillin assay.)
3. *Sterile human defibrinated blood* (10 cc.) inactivated at 50°C. for 30 minutes or kept in the refrigerator for 3 days.
4. *Test Organism.*
   a. A stock culture of hemolytic streptococcus (C-203) kept on blood agar and transferred every 2 weeks. (A culture may be obtained from Washington; see stock standard penicillin solution.)
   b. A 24 hour brain heart infusion broth (Difco) culture made from the stock culture is used in the test.
5. *Defibrinated Blood and Streptococcus Mixture.*
   a. Add the test organism to the blood in the proportion of 0.005 cc. of broth culture to 1 cc. of blood just before using in the test.
   b. The broth culture must be well mixed before pipetting.
6. *Stock Standard Penicillin Solution* (1 cc. = 100 units).
   a. Obtain sodium penicillin G crystals for assay and sensitivity tests from the Federal Security Agency, Food and Drug Administration, Washington, D. C. Keep in the refrigerator.
   b. Weigh the small bottle containing the crystals on an analytical balance.
   c. Pour out a small amount of the crystals (from 30 to 40 mg.) on a sterile watch glass and then reweigh the bottle to ob-

tain the weight of the amount poured out.

d. Dissolve the crystals in 100 cc. of sterile 0.85% NaCl solution and calculate the units per cc. (Use same technique as in making a standard solution for chemistry except use sterile glassware.)

e. Dilute in sterile 0.85% NaCl solution so that each cc. contains 100 units. (This solution may also be used for the sensitivity test.)

f. Example:
   1) Each mg. of the crystals contains 1667 units of penicillin.
   2) Weight of bottle before removing crystals minus weight of bottle after removing crystals equals 36.1 mg.
   3) In a 1-100 dilution each cc. would contain 601.787 units.

$$\left(\frac{36.1 \times 1667}{100}\right)$$

   4) A 1-6.02 dilution would equal 100 units per cc.

TABLE 35. DILUTIONS OF DILUTE STANDARD PENICILLIN SOLUTION

Tube	0.85% NaCl solution in cc.	Dilute standard penicillin solution in cc.	Dilution	Concentration of penicillin units per cc.
1	1.5	0.5	1-4	0.250
2	1.0	1 of 1-4	1-8	0.125
3	1.0	1 of 1-8	1-16	0.0625
4	1.0	1 of 1-16	1-32	0.03125
5	1.0	1 of 1-32	1-64	0.015
6	1.0	1 of 1-64*	1-128	0.0075

*Discard 1 cc. from this tube.

7. *Working Standard Penicillin Solution* (1 cc. = 10 units).
   a. Make a 1-10 dilution of the stock standard penicillin solution in sterile 0.85% NaCl solution and distribute in 1 to 2 cc. amounts in small vials.
   b. Keep frozen and allow to melt immediately before using.
8. *Dilute Standard Penicillin Solution* (1 cc. = 1 unit).
   a. Make a 1-10 dilution of the working standard penicillin solution in sterile 0.85% NaCl solution.
   b. This solution must be made fresh each day.

B. *Penicillin Standard Control.*

1. Dilution of dilute standard penicillin solution (1 cc. = 1 unit). See Table 35.
   a. Place 6 small sterile test tubes in a rack; add 1.5 cc. of sterile 0.85% NaCl solution to the first tube and 1 cc. to the remaining tubes.
   b. Add 0.5 cc. of the standard penicillin solution to the first tube and mix thoroughly.
   c. Transfer 1 cc. of this dilution to the second tube and mix.
   d. Transfer 1 cc. from the second tube to the third tube and so on through the sixth tube.
2. Transfer 0.2 cc. of the above dilutions respectively to 6 small sterile test tubes.
3. Add 0.2 cc. of the defibrinated blood and streptococcus mixture to each tube.
4. Shake and incubate the tubes at 37°C. overnight.
5. The reading of this test in concentration of penicillin units per cc. is used for the estimation of the amount of penicillin in the unknown fluid.

C. *Assay of Unknown Fluid.*

1. Place 6 small sterile test tubes in a rack and add 0.2 cc. of sterile 0.85% NaCl solution to all the tubes except the first. See Table 36.
2. Add 0.2 cc. of the fluid to be tested to the first and second tubes.
3. Mix the second tube, transfer 0.2 cc. to the third tube, etc., to the sixth tube and then discard 0.2 cc. from the sixth tube.
4. Add 0.2 cc. of the defibrinated blood and streptococcus mixture to each tube.
5. Shake and incubate the tubes at 37°C. overnight.

D. *Reading of Assay.*

1. The defibrinated blood is hemolyzed in the tubes containing growth of the hemolytic streptococcus, while there is no hemolysis in the tubes in which the organism is completely inhibited by penicillin.
2. The end-point is the last tube showing inhibition of growth of the organism, that is, the tube showing no hemolysis.
3. The end-point of the penicillin standard control should occur in tube 3 or 4, which contain concentrations of penicillin of 0.0625 and 0.03125 units per cc., respectively. This end-

point is the standard factor or the minimum concentration of the drug necessary to inhibit growth.

Read the end-point of the penicillin standard control and determine the standard factor (A), that is, the units per cc. which inhibits growth of the test organism.

Read the end-point of the unknown fluid and determine the dilution factor (B), that is, the highest dilution which inhibits growth of the test organism.

2 cc. of the working standard solution in a test tube and add 18 cc. of broth.

b. *Dilute standard B* (0.1 unit per cc.)—place 2 cc. of the dilute standard A solution in a test tube and add 18 cc. of broth.

5. *Organism to be Tested.*
   a. A 24 hour broth culture of the organism is necessary.
   b. Make a 1-10 dilution of the culture with broth.

TABLE 36. PENICILLIN ASSAY OF UNKNOWN FLUID

Tube	0.85% NaCl solution in cc.	Unknown fluid in cc.	Dilution	Defibrinated blood and streptococcus mixture in cc.
1	0	0.2	undil.	0.2
2	0.2	0.2	1-2	0.2
3	0.2	0.2 of 1-2	1-4	0.2
4	0.2	0.2 of 1-4	1-8	0.2
5	0.2	0.2 of 1-8	1-16	0.2
6	0.2	0.2 of 1-16*	1-32	0.2

iscard 0.2 cc. from this tube.

Calculation of penicillin concentration of the unknown fluid.

Standard factor (A) × dilution factor (B) = units per cc.

Example.
- a. Standard control test showed inhibition of hemolysis in first 3 tubes; therefore, the standard factor = 0.0625.
- b. The unknown fluid showed inhibition of hemolysis in first 4 tubes; therefore, the dilution factor = 1-8.
- c. 0.0625 × 8 = 0.5 unit per cc.

VII. Penicillin Sensitivity Test.

For Organisms sensitive to penicillin see Table 37.

Reagents Required.

Sterile Broth Culture Medium—use the kind of broth necessary for the best growth of the organism to be tested.

Stock Standard Penicillin Solution (1 cc = 100 units)–see penicillin assay.

Working Standard Penicillin Solution (1 cc. = 10 units).
   a. Make a 1-10 dilution of the stock penicillin standard solution in broth.
   b. Distribute in test tubes in 15 cc. amounts and freeze; avoid refreezing after once thawed due to loss of potency of the penicillin.

Dilute Standard Penicillin Solutions.
   a. *Dilute standard A* (1 unit per cc.)—place

C. Method.
1. Set up 15 sterile test tubes in a rack.
2. Add solutions according to Table 38.
3. Incubate tubes for 18 hours at 37°C.

D. Reading of Test.
1. The end-point is the first clear tube which indicates no growth of the organism.
2. This is the minimum concentration of penicillin necessary to inhibit the growth of the organism tested and is indicative of the sensitivity of the organism to penicillin.
3. For example, if tube 6 is the first clear tube, the organism is sensitive to penicillin in a concentration of 0.1 unit per cc.

VIII. Streptomycin Assay.

A. Reagents Required.
1. *Sterile 0.85% NaCl Solution.*
2. *Fluid to be Tested*—same as for penicillin assay.
3. *Sterile Human Defibrinated Blood* (10 cc.)—same as for penicillin assay.
4. *Test Organism.*
   a. A stock culture of Bacillus megatherium kept on a plain agar slant and transferred every 2 weeks. (A culture may be obtained from Washington; see stock standard streptomycin solution.)
   b. A 24 hour glucose broth culture is used in the test.
5. *Defibrinated Blood and B. megatherium Mixture.*

Table 37. Therapeutic Value of Penicillin, Streptomycin, and Sulfonamides

	Penicillin	Streptomycin	Sulfanilamide	Sulfapyridine	Sulfathiozole	Sulfadiazine	Sulfamerazine	Sulfaguanidine	Sulfasuxidine	Sulfathalidine
Optimum Blood Level Molecular Weight	*	*	10-15 mg.% 172	5-10 mg.% 249	4-10 mg.% 255	8-15 mg.% 250	10-15 mg.% 265	3-5 mg.% 236	3 mg.% 355	1.5 mg.% 403
Infection										
Streptococcus hemolyticus	++++	+	+++	+++	+++	++++	++++			
Streptococcus viridans	++	+	+	++	++	+	+			
Streptococcus (anaerobic)	++									
Staphylococcus	++++	++	+	+	++	++	++			
D. pneumoniae	++++	+	+	++	+++	+++	+++			
K. pneumoniae		+++		+	++	++	++			
N. gonorrhoeae	++++	+	+	+++	++++	++	++			
N. meningitidis	+++	+	++	+	+++	++++	++++			
H. influenzae		+++	+	+	++	++	++			
H. pertussis		+++								
C. diphtheriae	++	++++								
Coliform bacteria		++++		+	++	++	++	+	++	++++
Shigella group		+++	+	+	+	+	+	++	+++	++++
Salmonella group		++++				+				
S. typhosa		+				+				
Aerobacter aerogenes		++++				+				
Ps. aeruginosa		+++				+				
Brucella group		+				+				
P. tularensis		++++				+				
Proteus vulgaris		++++				+				
B. anthracis	++++	++++		++	+	+				
Clostridia	++++		+	+	++	+				++++
M. tuberculosis		+								
Actinomyces	++	+								
Treponema pallidum	+++	+								
Borrelia novyi	++	+								
Leptospira icterohaemorrhagiae	++	+								
Spirillum minus	++	+								
Streptobacillus moniliformis	++	+								

*Optimum blood level depends on sensitivity of organism.
+ = slight therapeutic value.
++++ = greatest therapeutic value.
Blank space = no therapeutic value.

TABLE 38. TEST FOR PENICILLIN SENSITIVITY

ube	Penicillin standard in cc.	Broth in cc.	Organism (1-10 dilution) in cc.	Concentration in units per cc.
1	0.5 (0.1 u/cc.)*	4.0	0.5	0.01
2	1.0 (0.1 u/cc.)	3.5	0.5	0.02
3	2.0 (0.1 u/cc.)	2.5	0.5	0.04
4	3.0 (0.1 u/cc.)	1.5	0.5	0.06
5	4.0 (0.1 u/cc.)	0.5	0.5	0.08
6	0.5 (1.0 u/cc.)	4.0	0.5	0.1
7	1.0 (1.0 u/cc.)	3.5	0.5	0.2
8	2.0 (1.0 u/cc.)	2.5	0.5	0.4
9	3.0 (1.0 u/cc.)	1.5	0.5	0.6
10	4.0 (1.0 u/cc.)	0.5	0.5	0.8
11	0.5 (10.0 u/cc.)	4.0	0.5	1.0
12	1.0 (10.0 u/cc.)	3.5	0.5	2.0
13	2.0 (10.0 u/cc.)	2.5	0.5	4.0
14	3.0 (10.0 u/cc.)	1.5	0.5	6.0
15	4.0 (10.0 u/cc.)	0.5	0.5	8.0

dard solution to be used is in parentheses.

Add the test organism to the blood in the proportion of 0.005 cc. of broth culture to 1 cc. of blood just before using in the test.

. The broth culture must be well mixed before pipetting.

tock Standard Streptomycin Solution (1 cc. = 100 micrograms or 0.1 mg.).

. Obtain streptomycin for assay and sensitivity tests from the Federal Security Agency, Food and Drug Administration, Washington, D. C. Keep in the refrigerator.

. Weigh out the streptomycin in the same manner as penicillin (see penicillin assay).

. Dissolve the streptomycin in 10 cc. of sterile 0.85% NaCl solution.

l. Dilute with sterile 0.85% NaCl solution so that each cc. will contain 100 micrograms or 0.1 mg.

. This solution will keep 1 month if frozen; 2 weeks if kept in the refrigerator not frozen.

Vorking Standard Streptomycin Solution (1 c. = 25 micrograms or 0.025 mg.)—dilute 1 cc. of the stock standard solution with 3 cc. of sterile 0.85% NaCl solution.

B. *Streptomycin Standard Control.*

1. Dilution of working standard streptomycin solution (25 micrograms per cc.). See Table 39.
   a. Place 6 small sterile test tubes in a rack and add 0.5 cc. of sterile 0.85% NaCl solution to each except the first.
   b. Add 0.5 cc. of the working standard streptomycin solution to the first and second tubes and mix the second tube thoroughly.
   c. Transfer 0.5 cc. of this dilution to the third tube and mix.
   d. Transfer 0.5 cc. of the third tube to the fourth and so on through the sixth tube.
2. Transfer 0.2 cc. of each of the above dilutions respectively to 6 small sterile test tubes.
3. Add 0.2 cc. of the defibrinated blood and B. megatherium mixture to each tube.
4. Shake and incubate at 37°C. overnight.
5. The reading of this test in micrograms of streptomycin per cc. is used for the estima-

TABLE 39. DILUTIONS OF WORKING STANDARD STREPTOMYCIN SOLUTION

Tube	0.85% NaCl solution in cc.	Working standard streptomycin solution in cc.	Dilution	Concentration of streptomycin in micrograms per cc.
1	0	0.5	undiluted	25.0
2	0.5	0.5	1-2	12.5
3	0.5	0.5 of 1-2	1-4	6.25
4	0.5	0.5 of 1-4	1-8	3.125
5	0.5	0.5 of 1-8	1-16	1.56
6	0.5	0.5 of 1-16*	1-32	0.78

ard 0.5 cc. from this tube.

TABLE 40. TEST FOR STREPTOMYCIN SENSITIVITY

Tube	Streptomycin standard in cc.	Broth in cc.	Organism (1-10 dilution) in cc.	Concentration in micrograms per cc.
1	0.5 (0.01 mg./cc.)*	4.0	0.5	1
2	1.0 (0.01 mg./cc.)	3.5	0.5	2
3	2.0 (0.01 mg./cc.)	2.5	0.5	4
4	3.0 (0.01 mg./cc.)	1.5	0.5	6
5	4.0 (0.01 mg./cc.)	0.5	0.5	8
6	0.5 (0.1 mg./cc.)	4.0	0.5	10
7	1.0 (0.1 mg./cc.)	3.5	0.5	20
8	2.0 (0.1 mg./cc.)	2.5	0.5	40
9	3.0 (0.1 mg./cc.)	1.5	0.5	60
10	4.0 (0.1 mg./cc.)	0.5	0.5	80

*Standard solution to be used is in parentheses.

tion of the amount of streptomycin in the unknown fluid.

C. *Assay of Unknown Fluid.*

1. Place 6 small sterile test tubes in a rack.
2. Add solutions in the same manner as given in Table 36 for assay of penicillin except add the defibrinated blood and B. megatherium mixture instead of the streptococcus mixture.
3. Shake and incubate the tubes at 37°C. overnight.

D. *Reading of Assay.*

1. B. megatherium produces hemolysis of erythrocytes.
2. The end-point of the standard streptomycin control and the unknown fluid is read in the same manner as in the penicillin assay.
3. The end-point of the streptomycin standard control should be in tube 3 or 4 which contains 6.25 and 3.125 micrograms per cc. respectively.
4. Calculation of the streptomycin concentration of the unknown fluid is made in the same manner as for penicillin using the appropriate standard factors.

IX. Streptomycin Sensitivity Test.

A. *For Organisms* sensitive to streptomycin see Table 37, page 188.

B. *Reagents Required.*

1. *Sterile Broth Culture Medium*—use the kind of broth necessary for the best growth of the organism to be tested.
2. *Stock Standard Streptomycin Solution* (1 cc. = 100 micrograms or 0.1 mg.).
   a. Weigh out the streptomycin and add 10 cc. of sterile 0.85% NaCl solution as described under stock standard streptomycin solution under streptomycin assay.
   b. Dilute with sterile broth so that each cc. will contain 100 micrograms or 0.1 mg.
   c. This solution will keep 1 month if frozen; 2 weeks if kept in the refrigerator not frozen.
3. *Dilute Standard Streptomycin Solution* (1 cc. = 10 micrograms or 0.01 mg.)—dilute 2 cc. of the stock standard solution with 18 cc. of broth.
4. *Organism to be Tested.*
   a. A 24 hour broth culture of the organism is necessary.
   b. Make a 1-10 dilution of the culture with broth.

C. *Method.*

1. Set up 10 sterile test tubes in a rack and add solutions according to Table 40.
2. Incubate tubes for 18 hours at 37°C.

D. *Reading of Test.*

1. The end-point is the first clear tube which indicates no growth of the organism.
2. This is the minimum concentration of streptomycin necessary to inhibit the growth of the organism tested and is indicative of the sensitivity of the organism to streptomycin.
3. For example, if tube 6 is the first clear tube, the organism is sensitive to streptomycin in a concentration of 10 micrograms per cc.

Quellung Reaction (Capsular Swelling)

I. Typing of Pneumococcus in Sputum.

A. *Collection of Specimen.*

1. The sputum should be collected in a clean, dry, and preferably sterile container before therapy is started.
2. It should come from the deep air passages and be relatively free from saliva and nasal secretions.
3. Pneumonia sputum usually contains some blood and tends to be thick and sticky.
4. If sputum has dried in the container, it may be resuspended in broth for examination provided it has not stood long enough to permit

the contaminating organisms to overgrow the pneumococci.

If possible, preservatives should not be used. However, if sputum is to be transported any distance requiring more than 5 hours, 0.5% formalin may be added.

Material may be obtained from infants and small children after inducing a cough by gently touching the posterior wall of the pharynx with a sterile swab and collecting any mucus present.

Place the swab in a tube containing 2 to 3 cc. of broth containing 1 or 2 drops of sterile rabbit blood, agitate, and incubate broth for 6 to 8 hours, no longer.

Typing.

Make a smear of the sputum and stain by Gram's method to determine the presence and relative number of pneumococci. If no organisms are present, typing is useless.

Mark 3 slides so each is divided in 2 parts, label the areas A, B, C, D, E, and F.

By means of a small loop measuring 1 mm. in diameter, place a small amount of sputum in each area.

If the sputum is extremely tenacious and thick, beat a portion of it with 0.85% NaCl solution or broth by means of a wooden applicator in a Petri dish, using the flecks that float to the top for the reaction.

With a large loop measuring 4 mm. in diameter, place 4 loopfuls of rabbit serum A in the area with that letter. Place the serum at the edge of, but not touching, the sputum so that the loop is not contaminated and can be used for more serum without contaminating that in the bottle.

When the last drop of serum is added, mix serum thoroughly with sputum. If there is not enough methylene blue in the serum to stain the organisms, add one drop of Loeffler's methylene blue solution and mix again. Place a cover glass over the preparation.

Add each group serum to its corresponding area (B, C, D, E, and F) in the same manner, flaming the loop between each group serum.

8. Examine with a microscope, oil immersion lens, with a subdued light. Thin specimens may be examined immediately, but 30 minutes should elapse before looking at thick specimens. The thicker the specimen the more time should be allowed before a negative report is given.
9. A positive reaction is indicated by the appearance of a sharply outlined but colorless swollen capsule surrounding the blue-stained pneumococci. This swollen capsule has a distinct "ground glass" appearance. The degree of swelling is usually equivalent to the width of the pneumococcus, except in Type III it is much greater. The most significant thing is the sharpness of the capsular outline which is easily visible at different focal planes.
10. When large numbers of organisms are present, they are often agglutinated by the serum causing the individual capsules to be indistinguishable, except at the outer portion of the clump. A capsule-like film with a "ground glass" effect appears to surround the entire clump. This is especially true of Type III.
11. Examine all groups (A, B, C, D, E, and F) and repeat the same procedure with the individual types in the groups showing capsular swelling. There may be several different types present.
12. When several types are present, some indication as to the causative agent may be obtained from the relative proportion of the different types and by the incidence of different types in healthy individuals. The following types are commonly found in normal throats, III, VI, XIX, and XXIII.
13. There are a few unclassified pneumococci; if there is a strain present which does not react with any typing serum, culture and test its solubility in bile. Report as unclassified pneumococci if bile soluble.

C. *Lockard's Concentration Method.*

1. If there are only a few pneumococci on the Gram stain, proceed with the concentration method before typing.
2. Place approximately 0.5 gm. of caroid (obtained from American Ferment Co., Buffalo, N. Y.) in 8 cc. of 0.85% NaCl solution and mix well.
3. Pour the caroid-saline mixture over the sputum.
4. Stir well with a wooden applicator and allow to stand 5 minutes.
5. Place in a centrifuge tube and centrifuge at low speed for exactly 2 minutes to throw down the coarse particles and mucous threads.
6. Pour the supernatant fluid into another centrifuge tube and discard the sediment.
7. Centrifuge the fluid again for 15 minutes at high speed and decant the supernatant fluid.
8. Mix the sediment by shaking and use for typing as described above.

D. *Mouse Inoculation.*

1. Inoculate 0.5 cc. of the sputum into the peri-

toneal cavity of a mouse. If it is very thick and tenacious, add 1 cc. of sterile 0.85% NaCl solution, mix, and inoculate 0.5 to 1 cc. of the thinner mixture.

2. Observe the animal frequently; symptoms may appear after 4 to 5 hours.
3. When symptoms appear, or after 18 hours, puncture the peritoneum using a tuberculin syringe with a 25 gauge needle.
4. Withdraw some fluid, holding the mouse with the abdomen down.
5. If only a drop or two of fluid is obtained, mix with a few drops of sterile 0.85% NaCl solution.
6. Make a smear with a small loopful of the fluid and stain by Gram's method; if pneumococci are present, type as described for sputum.
7. If the mouse dies, use the peritoneal exudate (wash the peritoneal cavity with 2 to 3 cc. of 0.85% NaCl solution if necessary) for typing and culture the heart's blood on a blood agar plate and in broth.
   a. Pneumococci will appear as very small, round, flat, moist, water clear, nonconfluent colonies on the blood agar. The colonies will be surrounded by a zone of methemoglobin formation with an exterior zone of greenish color after 24 hours.
   b. Use the broth culture for a bile solubility test.

II. Typing of Pneumococcus in Other Fluids.

A. *Spinal Fluid.*

1. Centrifuge, make a smear of the sediment, and stain by Gram's method.
2. If pneumococci are present, proceed to type as described under sputum.
3. If more than 15 to 18 organisms are present per oil immersion field, dilute with an equal amount of sterile 0.85% NaCl solution.

B. *Material from Lung Puncture or Empyema Pus.*

1. Make a smear of the material and stain by Gram's method.
2. If a few pneumococcus organisms are present, proceed to type as described under sputum.
3. If more than 15 to 18 organisms per oil immersion field are present, dilute by mixing thoroughly a loopful of the material in 3 or 4 drops of sterile 0.85% NaCl solution.

C. *Cultures.*

1. Broth cultures or organisms from solid media suspended in 0.85% NaCl solution may be typed.
2. Proceed in the same manner as for sputum, diluting if the organisms are too numerous.

III. Typing of K. pneumoniae (Friedländer's Bacillus).

A. *Sputum.*

1. Use typing serums for Type A and Type B and proceed as in the method for pneumococcus typing.
2. Typing serums for Type C and Type X are not available.

B. *Cultures,* spinal fluid, or other material containing Friedländer's bacillus may be typed in the same manner.

IV. Typing of N. meningitidis.

A. *Spinal Fluid.*

1. If enough organisms are present, typing can be made directly on the sediment by the same method as described under pneumococcus typing but using meningococcus typing serums for Group I and Group II alpha.
2. Group II does not have demonstrable capsules.
3. Group I is found in 70% of the cases of meningococcus meningitis.

B. *Cultures.*

1. Add 0.05 cc. of a 1-10 saline dilution of a broth culture to 0.25 cc. of Group I and Group II alpha typing serum, respectively.
2. Incubate for 2 to 4 hours in an atmosphere of 10% CO_2.
3. Place a loopful on a slide with a loopful of methylene blue stain.
4. Look for capsular swelling with the oil immersion objective of the microscope.

V. Typing of H. influenzae.

A. *Spinal Fluid.*

1. If enough organisms are present, typing can be made directly on the sediment by the same method as described in the method under pneumococcus typing but using typing serum for H. influenzae, Type B.
2. Type B is found in almost all cases of influenzal meningitis.

B. *Cultures* and other material containing H. influenzae may be typed.

Agglutination Tests for Bacterial Antibodies

I. All tests in which living cultures are used must be carried out with the utmost care against self-infection and contaminating the laboratory with the organisms. Cultures of P. tularensis and the Brucella group are especially dangerous. Killed organisms as antigens are recommended.

II. Materials Required.

ient's Serum.

ain 5 cc. of venous blood and allow to t.

trifuge to separate serum.

ts may be run on blood that has stood 2 to 48 hours in the refrigerator.

tigen prepared as described below.

dium Chloride Solution—0.85%.

all Test Tubes about 8 mm. in diameter.

III. Preparation of Antigens.

spensions of Living Bacteria—12 to 24 ur living plain broth culture; a nonmotile ing culture of Proteus is best for the Weil-lix reaction.

eparation of H (Flagellar) Antigens.

lagellar antigens are used in detecting H gglutinins against S. typhosa, S. paratyphi A), S. schottmülleri (para B), and S. choeraesuis.

n actively motile, smooth colony strain is ssential for each species.

he motility may be enhanced by growing he culture either in broth or on agar at oom temperature for a few days.

Streak a plate of beef infusion agar poured 24 hours previously and incubate for 18-24 hours at 37°C.

Examine for smooth colonies; if all the colonies are not smooth, suspend 2 or 3 smooth colonies in small amounts of saline or broth and replate each suspension immediately.

Repeat this process until all the colonies are smooth.

Make a smear, stain by Gram's method, and examine to ascertain whether the culture is pure.

Inoculate an agar slant and incubate for 18 to 24 hours.

Suspend the culture in saline or broth and inoculate Kolle flasks or Blake bottles containing 2% beef infusion agar from which the water of condensation has been removed with a pipette.

Incubate 18-24 hours at 37°C. and then pipette 10 cc. of sterile buffered NaCl solution containing 2% formalin into the flask to suspend the growth.

Transfer to a sterile test tube and place in the refrigerator for 48 hours.

Culture for sterility by pipetting 0.1 cc. of the suspension into a tube of thioglycollate glucose broth and incubating.

If not sterile, repeat culture every 48 hours until sterile.

14. If considerable material settles out, decant the supernatant suspension and dilute it with buffered NaCl solution to a turbidity 10 times heavier than tube No. 3 of McFarland's nephelometer.
15. For agglutination tests, combine 1 part of the concentrated suspension with 9 parts of sterile 0.85% NaCl solution containing 0.2% formalin to give a turbidity comparable to tube No. 3.
16. Test for agglutination with a known positive serum.
17. Store in the refrigerator.
18. *Buffered NaCl Solution.*

Sodium chloride	6.8 gm.
Sodium phosphate (Na_2HPO_4)	2.0 gm.
Water to make 1 liter.	

   a. Check pH which should be 8.4 - 8.6.
   b. Filter through Whatman No. 50 filter paper.
   c. Autoclave at 15 lbs. pressure for 30 minutes.

C. ***Preparation of O (Somatic) Antigen.***

1. Somatic antigens are used in detecting O agglutinins against S. typhosa, Proteus X_2, Proteus X_{19}, and Proteus XK.
2. Follow directions under preparation of H antigen for obtaining a smooth colony strain and inoculation of Kolle flasks or Blake bottles. (It may be either a motile or nonmotile strain except for Proteus which must be a nonmotile strain.)
3. After incubating for 18-24 hours, suspend the growth in 10 cc. of sterile 0.85% NaCl solution containing 0.5% phenol.
4. Pour into a large test tube or flask and add with constant shaking an equal quantity of absolute alcohol.
5. Incubate at 37°C. for 12-18 hours.
6. Decant the supernatant suspension into a sterile test tube or flask.
7. Culture for sterility by pipetting 0.1 cc. of the suspension into a tube of thioglycollate glucose broth.
8. Add one-half volume of sterile 0.85% NaCl solution containing 0.5% phenol to reduce the concentration of alcohol to approximately 30%.
9. Further dilute the suspension with sterile 0.85% NaCl solution containing 0.5% phenol combined with 30% of its volume of absolute alcohol until the turbidity is approximately 10 times that of tube No. 3 of McFarland's nephelometer.
10. For agglutination tests, combine 1 part of the concentrated suspension with 9 parts of 0.85% NaCl solution to obtain a suspension containing approximately 3% alcohol and

0.05% phenol and equivalent in turbidity to tube No. 3.

11. Test for agglutination with a known positive serum.
12. Store in the refrigerator.

D. ***Preparation of Shigella Antigens.***

1. Although Shigella organisms are not motile, the antigens are prepared as described under H antigens.
2. If the antigen tends to agglutinate spontaneously, use a 2% formalized 0.5% NaCl solution instead of a formalized 0.85% solution for washing off the growth from the agar.

E. ***Preparation of Brucella Antigens.***

1. Make antigens of Brucella abortus, suis, and melitensis. (Cultures may be obtained from the National Institute of Health, Bethesda, Md.)
2. Caution must be taken in handling virulent strains.
3. Ascertain whether the strain produces smooth colonies in the same manner as described under the preparation of H antigens.
4. Inoculate Kolle flasks or Blake bottles containing Bacto-tryptose agar with a suspension of a 72 hour culture.
5. Incubate 72 hours at 37°C. and wash off the growth with 0.5% phenol in 0.85% NaCl solution.
6. Filter through several layers of sterile gauze into a sterile vaccine bottle.
7. Refrigerate the suspension for 7 to 10 days and make a sterility test.
8. This is the stock solution and keeps indefinitely.
9. For use in the test, dilute with phenolized saline solution so the suspension is comparable to tube No. 1 of McFarland's nephelometer.

F. ***Preparation of P. tularensis Antigen.***

1. A nonvirulent strain of P. tularensis must be used. (A nonvirulent strain "B-38" may be obtained from the National Institute of Health, Bethesda, Md.)
2. Wash off the growth from a 48-72 hour culture on a glucose-cystine blood agar slant with 1 cc. of sterile 0.85% NaCl solution.
3. Inoculate this suspension onto the surface of glucose-cystine blood agar in a Kolle flask or Blake bottle.
4. Incubate 72 hours at 37°C.
5. Wash off with 15 cc. of 0.5% formalin in 0.85% NaCl solution and place in a 50 cc. centrifuge tube.
6. Centrifuge, pour off the supernatant fluid, and resuspend in the same amount of formalized saline solution.
7. Place in the refrigerator for 7 to 10 days,
for sterility.
8. This is the stock antigen.
9. Dilute for the test with formalized saline
the suspension is comparable to tube No. 4
McFarland's nephelometer.

IV. Macroscopic Agglutination Test.

A. ***Method***—see Table 41.

1. Place 10 small test tubes in a rack and add
each one, except the first, 0.5 cc. of 0.85
NaCl solution.
2. Make a 1-10 dilution of the patient's seru
with the NaCl solution and add 0.5 cc. to th
first and second tubes only.
3. Shake the second tube and with a pipett
transfer 0.5 cc. to the third tube.
4. Shake this tube and transfer 0.5 cc. to th
fourth tube and so on through the ninth tub
from which 0.5 cc. is discarded.
5. The tenth tube serves as a control and con
tains only 0.5 cc. of NaCl solution withou
any serum.
6. Add 0.5 cc. of antigen to each tube.
7. Mix all the tubes thoroughly by shaking.

B. ***Incubation Period.***

1. Tests using H antigens should be incubated
hours at 52°C. and then placed in the refriger
ator overnight before reading.
2. Tests using "O" antigens should be incubate
overnight at 52°C. and then read.
3. Tests using Brucella antigens should be incu
bated for 48 hours in a 37°C. incubator.
4. Tests using P. tularensis antigen and Proteu
antigens should be incubated for 2 hours
37°C. and placed in the refrigerator over
night before reading.
5. Tests for Shigella, Salmonella, and Brucell
may be shaken for 6 minutes in the sam
manner as for Kahn tests and then incubate
for 4 hours at 37°C. The test can either b
read immediately after centrifuging the tube
a few minutes at low speed or after placin
them in the refrigerator overnight.

C. ***Reading of Test.***

1. Observe every tube for clearing of the supe
natant fluid.
2. Shake the tube gently and observe the amou
and character of agglutinated particles.
agglutinins produce floccular clumps whic
are easily broken up, while O agglutinins pr
duce granular clumps.
3. Complete agglutination with complete clea
ing of the supernatant fluid indicates a 4
reaction. Decreasing amounts of aggluti
tion and increasing cloudiness of the supe

TABLE 41. AGGLUTINATION TEST FOR BACTERIAL ANTIBODIES

ube	0.85% NaCl solution in cc.	Serum in cc.	Antigen in cc.	Final dilution of serum
1		0.5 of 1-10	0.5	1-20
2	0.5	0.5 of 1-10	0.5	1-40**
3	0.5	0.5 of 1-20	0.5	1-80
4	0.5	0.5 of 1-40	0.5	1-160
5	0.5	0.5 of 1-80	0.5	1-320
6	0.5	0.5 of 1-160	0.5	1-640
7	0.5	0.5 of 1-320	0.5	1-1280
8	0.5	0.5 of 1-640	0.5	1-2560
9	0.5	0.5 of 1-1280*	0.5	1-5120
(Control)	0.5		0.5	

ard 0.5 cc. from this tube.

l dilution is 1-40 because 0.5 cc. is transferred from Tube 2 to Tube 3 leaving 0.5 cc. of the serum-saline e in Tube 2.

tant fluid are read as 3, 2, and 1 plus re- tions.

he control tube and the negative tubes will e cloudy without any agglutination.

V. Interpretation of Results.

egative Results.

atient does not have infection for which the est was made.

he sample of blood was taken before the ppearance of agglutinins in the serum. See able 42 for appearance of agglutinins in arious diseases.

negative test followed within a week or 0 days by repeated positive tests with a pro- ressively increasing agglutination titer is defi- ite evidence of active infection.

ositive Results.

yphoid Fever.

. Indication of a definite present infection.
  1) Three or 4 plus in a 1-80 or higher dilution with O antigen.
  2) Three or 4 plus in a 1-80 or higher dilution with H antigen.

. Indication of a past infection, recent vaccination, or of a carrier.
  1) Three or 4 plus in a 1-80 or higher dilution with H antigen.
  2) Little or no agglutination with O antigen.

ysentery—a positive reaction of 1-80 is con- idered significant.

rucellosis—a positive reaction of 1-40 or ver suggests infection, past or present. An ncrease in titer of Brucella agglutinins occurs or several years after vaccination for cholera nd may result in an erroneous diagnosis of rucellosis.

ularemia.

. A positive reaction of 1-40 or over suggests definite infection.

b. Cross agglutination with the Brucella group must be ruled out.

5. *Rickettsial Diseases.*
   a. Agglutination in a dilution of 1-80 is suspicious, while 1-160 is considered positive.
   b. This is the Weil-Felix reaction using antigens of Proteus OX_{19}, Proteus OX_2, and Proteus OXK.
   c. For routine tests Proteus OX_{19} is usually sufficient.
   d. Typhus fever (epidemic and murine) agglutinins usually give a strongly positive test with only OX_{19}, a weak agglutination with OX_2, and none with OXK.
   e. Rocky Mountain spotted fever agglutinins will give either a strong agglutination with OX_{19} and a weak reaction with OX_2, or a weak agglutination with OX_{19} and a strong reaction with OX_2; the agglutinins do not react with OXK.
   f. Scrub typhus (Tsutsugamushi fever) agglutinins will give a strong agglutination with OXK only.
   g. "Q" fever agglutinins will not agglutinate the three antigens.
   h. There may be cross-agglutination with the Brucella group.

Agglutination Test for Heterophil Antibodies

I. **Agglutinins for sheep cells** are increased in infectious mononucleosis and serum sickness. These are called heterophil antibodies because they are produced by an antigen which, when injected into certain animals, will elicit not only specific antibodies but also nonspecific antibodies; the presence of the latter is demonstrable by their reaction with antigens other than those involved in their production. For example, tissues of guinea pigs, horses, mice, and chickens, as well as human erythrocytes of group A and group AB, contain a protein in

TABLE 42. INTERPRETATION OF AGGLUTINATION TESTS

	Appearance of agglutinins	Maximum height of agglutinins	Positive titer
Typhoid	7-10 days	3-5 weeks	1-80
Paratyphoid (Salmonella group)	7-10 days	3-5 weeks	1-80
Dysentery (Shigella group)	2nd week	2-3 weeks	1-80
Brucellosis	2nd week	3-6 weeks	1-40
Tularemia	4-5 days	4-7 weeks	1-40
Rickettsial diseases	4-5 days	1-2 weeks	1-160
Infectious mononucleosis	4-28 days	At height of fever	1-112

combination with lipoid and polysaccharide haptens capable of producing an agglutinin and hemolysin for sheep corpuscles when injected into rabbits. The agglutinins for sheep cells in infectious mononucleosis can be differentiated from those in normal serum and from those in serum of patients with either serum sickness or who have recently been injected with horse serum by absorption tests with kidney tissue of guinea pigs and beef erythrocytes, see Table 43.

TABLE 43. AGGLUTININS FOR SHEEP ERYTHROCYTES IN HUMAN SERUM

Type of serum	Absorbed by guinea pig kidney	Absorbed by beef erythrocytes
Normal	+	−
Infectious mononucleosis	−	+
Serum sickness	+	+

II. Davidsohn's Method.

A. *Materials Required.*

1. *Patient's Serum.*
   a. Obtain serum as described for the Wassermann test, page 226.
   b. Inactivate in a water bath at 56°C. for 30 minutes.
2. *Sodium Chloride Solution—0.85%.*
3. *Sheep Corpuscle Suspension—2%.*
   a. Cells must be at least 24 hours old and not more than 1 week old.
   b. Wash the cells 3 times with 0.85% NaCl solution on the day they are to be used in the same manner as for the Wassermann test, page 222.
   c. The supernatant fluid after the third centrifugation must be clear and colorless.
   d. Make a 2% suspension of the thoroughly packed cells in 0.85% NaCl solution.

B. *Method.*

1. Place 12 test tubes, about 10 by 75 mm., in a rack and add 0.4 cc. of 0.85% NaCl solution to the first tube and 0.25 cc. to the remaining 11 tubes; see Table 44.
2. Add 0.1 cc. of inactivated serum to the first tube and mix thoroughly.
3. Transfer 0.25 cc. of the mixture to the secor tube and mix.
4. Continue to tube 11 and discard 0.25 cc. the mixture from this tube.
5. Tube 12 serves as a control containing on 0.25 cc. of NaCl solution.
6. Add 0.1 cc. of a 2% sheep cell suspensio to each of the 12 tubes.
7. Shake the tubes and leave at room temper ture for 2 hours.
8. Shake the tubes gently until the entire sec ment is suspended and report the amount agglutination as follows:

   + + + = cells remain in the form of a single clum
   + + = cells break up into distinctly visil clumps and the fluid is clear and tra parent.
   + = fine clumps of cells when the test tube placed horizontally on the stage of microscope and observed with the lo power objective.

C. *Interpretation.*

1. The following titers are for final dilutior
2. *Normal*—a titer of 1-28 with an occasion 1-56.
3. *Serum sickness* may give titers as high 1-224.
4. *Recent injections of horse serum* without s rum sickness may give a titer of 1-56 or 1-11
5. *Infectious mononucleosis.*
   a. A titer of 1-56 is suspicious.
   b. A titer of 1-112 in a person, who giv no history of having received injectio of horse serum in the recent past, in probability indicates the person has infe tious mononucleosis if he presents th clinical and hematological findings.
   c. A titer of 1-224 is considered positi even if there is a history of administr tion of horse serum, unless the patient suffering with serum disease or has ve recently recovered from it.
   d. A small percentage of cases of infectio mononucleosis have a normal titer.
6. The serum of patients with lymphatic leuk mia show no agglutination in titers above 1-

TABLE 44. AGGLUTINATION TEST FOR HETEROPHIL ANTIBODIES

Tube	0.85% NaCl solution in cc.	Serum in cc.	cc. of 2% sheep cells	Final dilution of serum
1	0.4	0.1	0.1	1-7**
2	0.25	0.25 of 1-5	0.1	1-14
3	0.25	0.25 of 1-10	0.1	1-28
4	0.25	0.25 of 1-20	0.1	1-56
5	0.25	0.25 of 1-40	0.1	1-112
6	0.25	0.25 of 1-80	0.1	1-224
7	0.25	0.25 of 1-160	0.1	1-448
8	0.25	0.25 of 1-320	0.1	1-896
9	0.25	0.25 of 1-640	0.1	1-1792
10	0.25	0.25 of 1-1280	0.1	1-3584
11	0.25	0.25 of 1-2560*	0.1	1-7168
12 (Control)	0.25		0.1	

*Discard 0.25 cc. from this tube.

**Final dilution is 1-7 because 0.25 cc. is transferred from Tube 1 to Tube 2 leaving 0.25 cc. of the serum-saline ixture in Tube 1.

II. Absorption Tests to Differentiate Types of Heterophil Agglutinins.

. *Guinea Pig Kidney Antigen.*

. The kidneys of guinea pigs may be kept frozen in the refrigerator until needed.

. They are thawed, cut into large pieces, and washed repeatedly with 0.85% NaCl solution until the washings are free of blood.

3. Place in a mortar and mash to a fine pulp.

4. Make a 20% suspension in 0.85% NaCl solution and boil for 1 hour in a water bath.

5. Make up the loss of fluid by evaporation with distilled water.

6. Add enough phenol to make a 0.5% solution and the suspension will keep many months in the refrigerator.

B. *Beef Corpuscle Antigen.*

1. Wash defibrinated beef corpuscles 3 times with 0.85% NaCl solution in the same manner as washing sheep cells for the Wassermann test, page 222.

. Make a 20% suspension of the packed cells by adding 4 volumes of 0.85% NaCl solution and boil for 1 hour in a water bath.

. Make up the loss of fluid by evaporation with distilled water.

. Add enough phenol to make a 0.5% solution and the suspension will keep many months in the refrigerator.

C. *Absorption.*

. Place 1 cc. of the 20% suspension of guinea pig kidney in 1 test tube and 1 cc. of the 20% beef corpuscle suspension in another test tube.

. Add 0.2 cc. of the patient's serum which has been inactivated for 30 minutes at 56°C. to each tube.

. Shake and let stand at room temperature for 1 hour, shaking at 15 minute intervals.

4. Centrifuge at 1500 revolutions per minute for 10 minutes and remove the supernatant fluid with a capillary pipette; this is a 1-5 dilution of the serum.

5. Repeat the test for heterophil antibodies as described under Davidsohn's method for each antigen, except use the above absorbed serum as follows:
   a. Place 0.25 cc. of the 1-5 serum dilution in the first tube which should not contain any NaCl solution.
   b. Place 0.25 cc. of the 1-5 serum dilution in the second tube which contains 0.25 cc. of NaCl solution, mix, and transfer 0.25 cc. to the third tube, etc.

D. *Interpretation.*

1. The guinea pig kidney antigen will not absorb the agglutinins found in infectious mononucleosis so the titer will remain high; however, it may be one-fourth lower than with unabsorbed serum due to the absorption of the normal agglutinins for sheep cells.
2. If all the agglutinins are removed, the disease in question is not infectious mononucleosis.
3. The beef corpuscle antigen will absorb almost all the agglutinins for sheep cells so the titer should not be more than that found with normal serum. This is only a confirmatory test.
4. See Table 43.

Vaccines and Filtrates

I. Autogenous Vaccine.

A. *Obtaining the Bacteria.*

1. Make a smear of the material from the patient's lesion and stain by Gram's method.
2. Streak the material on a blood agar plate or

on other media in plate form depending on the organisms found in the smear.

3. Incubate 24 hours.
4. Pick typical colonies, make smears, and stain by Gram's method to determine the kind of organisms.
5. Obtain each organism in pure culture by streaking out on a plate.
6. Pick smooth colonies of each organism present and make 4 glucose agar slants or broth cultures depending on which the organisms grow best.

B. *Making Suspension.*

1. *From Agar Slants.*
   - a. Add a few cc. of sterile 0.85%NaCl solution to each of the agar slants to wash off the growth and, if necessary, rub off the growth with a platinum loop.
   - b. Transfer the washings from all 4 agar slants to a sterile 50 cc. centrifuge tube and centrifuge for 30 minutes at high speed.
   - c. Pour off the supernatant fluid, add 2 or 3 sterile beads, and shake thoroughly to break up all the clumps.
   - d. Dilute to 10 cc. with sterile 0.85% NaCl solution.
2. *Broth Cultures.*
   - a. Pour contents of each of the 4 cultures into a sterile 50 cc. centrifuge tube and centrifuge for 30 minutes at high speed.
   - b. Pour off the broth and add sterile 0.85% NaCl solution to the sediment and mix.
   - c. Centrifuge and pour off the supernatant fluid.
   - d. Add 2 or 3 sterile glass beads and shake to break up all the clumps.
   - e. Dilute to 10 cc. with sterile 0.85% NaCl solution.

C. *Sterilization.*

1. *Chemical Method.*
   - a. Add 1 cc. of 5% phenol solution per 10 cc. of concentrated bacterial suspension (15-20 billion per cc.).
   - b. Allow to stand at room temperature for 3 days.
   - c. Culture for sterility by pipetting 0.1 cc. of the suspension into a tube of thioglycollate glucose broth.
   - d. If no growth appears after 72 hours incubation, dilute to the desired number of bacteria by adding 0.5% phenolized 0.85% NaCl solution.
   - e. Transfer to a sterile vaccine bottle and culture after 24 hours as above.
2. *Heat Method.*
   - a. Heat the bacterial suspension in a water bath at 58°C. for 1 hour.
   - b. Culture for sterility by pipetting 0.1 cc. of the suspension into a tube of thioglycollate glucose broth and incubate for 72 hours.
   - c. In the meantime keep the bacterial suspension in the refrigerator.
   - d. If there is growth in the broth, the suspension must be placed in the water bath again for 1 hour and cultured as above.
   - e. If no growth is found, add 1 cc. of 5% phenol solution and then standardize the suspension to the desired number of bacteria by adding 0.5% phenolized 0.85% NaCl solution.
   - f. Transfer to a sterile vaccine bottle and culture after 24 hours as above.
3. *Combination Heat and Chemical Method.*
   - a. Add 1 cc. of 5% phenol solution to the bacterial suspension and heat as described under heat method for 30 minutes.
   - b. Place in the refrigerator for 4 days and then culture for sterility as described under the other 2 methods.
   - c. If not sterile, culture again at the end of 1 week.
   - d. When sterile transfer to a vaccine bottle and culture after 24 hours as above.

D. *Standardization.*

1. *Counting Method.*
   - a. Make an erythrocyte count on the oxalated blood to be used.
   - b. Place a wax pencil mark about 2 inches from the tip of a capillary pipette.
   - c. Draw up the following consecutively in the pipette.
     - 1) Oxalated blood to the pencil mark and then air for about 5 to 10 mm.
     - 2) Bacterial suspension to the pencil mark and then air for about 5 to 10 mm.
     - 3) NaCl solution (0.85%) to the pencil mark and then air the length of the capillary portion of the pipette.
   - d. Mix this suspension thoroughly in the pipette above the capillary portion.
   - e. Make 3 or 4 smears of the suspension and stain with Wright's stain.
   - f. Place a Whipple eyepiece micrometer in the microscope. Instead of a micrometer, a piece of paper the size of the eyepiece and with an opening 5 mm. square cut in the center may be placed in the eyepiece. This reduces the size of the field.
   - g. Count the number of erythrocytes and bacteria in 20 fields (or until 1000 eryth-

rocytes are counted) and determine the number of organisms per cc. of vaccine.

Calculation:

$$\text{No. bacteria per cc.} = \frac{a \times b}{c}$$

a = No. of bacteria counted in 20 fields.
b = No. of erythrocytes in each cc. of oxalated blood (erythrocyte count times 1000 to obtain number per cc.).
c = No. of erythrocytes counted in 20 fields.

Dilute organisms with sterile 0.85% NaCl solution containing 0.5% phenol to make the proper suspension.

urbidity Method.

The correct suspension is obtained by diluting with sterile 0.85% NaCl solution containing 0.5% phenol until the cloudiness compares with known standards of killed organisms or with McFarland's nephelometer.

Standards of Killed Organisms.

1) These standards are made of killed organisms that have been counted as described under 1.
   Staphylococcus—5 billion per cc.
   Streptococcus and other organisms—1 billion per cc.
2) They should be sealed in test tubes of the same diameter as the tubes used for diluting the vaccine.

McFarland's Nephelometer.

1) Arrange 10 large test tubes, the same size as used in diluting vaccines, in a rack and label 1 to 10.
2) Add a 1% solution of anhydrous barium chloride and a 1% (by volume) cold solution of chemically pure sulfuric acid according to Table 45.
3) Seal tubes and keep in refrigerator.
4) When the fine white precipitate of barium sulfate is shaken up well, each tube has a different density corresponding approximately to bacterial suspensions as given in Table 45.

Table 45. McFarland's Nephelometer

Barium chloride (1%) in cc.	Sulfuric acid (1%) in cc.	Corresponding bacterial suspension per cc.
0.1	9.9	300,000,000
0.2	9.8	600,000,000
0.3	9.7	900,000,000
0.4	9.6	1,200,000,000
0.5	9.5	1,500,000,000
0.6	9.4	1,800,000,000
0.7	9.3	2,100,000,000
0.8	9.2	2,400,000,000
0.9	9.1	2,700,000,000
1.0	9.0	3,000,000,000

II. Sputum Vaccine.

A. ***Isolate organisms*** as described under sputum culture.

B. ***Make a vaccine of each organism*** as described under autogenous vaccine.

1. The vaccine of each organism may be kept separate and used in this manner.
2. The vaccines of the various organisms may be mixed in the proportion in which they occurred in the original smear of the sputum and used as a mixture.

III. Bacterial Filtrate (Bacterial Autolysate).

A. ***Preparation.***

1. Inoculate 200 cc. of glucose broth with a loopful of a pure culture of the organism.
2. Incubate at 37°C. for 6 days.
3. Add 1 cc. of melted phenol, drop by drop, with continual shaking. This makes a 0.5% solution of phenol.
4. Place in the refrigerator overnight, pass through a sterile Seitz filter, and transfer to sterile vaccine bottles.

B. ***Test for Sterility.***

1. After standing 3 or 4 days, remove 0.2 cc. with a sterile needle and syringe and place in thioglycollate glucose broth.
2. Incubate for 72 hours and if there is no growth the filtrate may be used.
3. If growth appears, the filtrate must be discarded and a new autolysate must be prepared.

Guinea Pig Inoculation for Tubercle Bacilli

I. Pus.

A. ***Inoculation.***

1. Always make 2 smears of the pus first; stain 1 with Loeffler's methylene blue solution immediately to ascertain whether or not there are many bacteria present and stain the other smear by the Ziehl-Neelsen method for acid-fast organisms.
2. If no bacteria are found with the methylene blue stain, inject 1 cc. subcutaneously in the groin of a guinea pig weighing about 300 gm. and 1 cc. intraperitoneally.
3. If there are a few organisms present, inject 1 cc. subcutaneously only.
4. If the pus is very thick, it can be diluted with sterile 0.85% NaCl solution before injecting.
5. Weigh the guinea pig.
6. If the guinea pig dies in less than 2 months, weigh and autopsy.

7. If the pig does not die in 2 months, kill, weigh, and autopsy.

B. *Positive Autopsy Findings.*

1. Enlarged caseous lymph glands are found in the groin on the side of the inoculation.
2. Multiple tubercles up to 2 or 3 mm. in diameter are found in the spleen and usually in the liver.
3. The spleen is usually 2 to 3 times normal size.
4. Enlarged caseous lymph glands are often found in the omentum.
5. Make smears of the cut surface of the caseous lymph glands and the spleen.
6. Stain the smears by the Ziehl-Neelsen method and examine for tubercle bacilli.

II. Urine.

A. *Inoculation.*

1. Urine should be a catheterized specimen and must be kept free from contamination.
2. Centrifuge the entire specimen in 50 cc. sterile centrifuge tubes at a high rate of speed for 45 minutes. If the specimen is small and appears concentrated, dilute with sterile distilled water to a pale straw color before centrifuging.
3. Remove the upper 1 cc. layer with a sterile pipette, decant the supernatant fluid, and add the urine in the pipette to the sediment and mix.
4. Make 2 smears of the sediment; stain 1 with Loeffler's methylene blue solution immediately to see if there are any other bacteria present and stain the other smear by the Ziehl-Neelsen method for acid-fast organisms.
5. If no bacteria are found with the methylene blue stain, inject part of the sediment subcutaneously into the groin of a guinea pig weighing about 300 gm. and the remainder intraperitoneally.
6. If there are many bacteria present, digest with 5% oxalic acid as described under urine culture for tubercle bacilli on page 178 and then inject subcutaneously into the groin of a guinea pig.
7. Weigh the guinea pig.

B. *Autopsy.*

1. If the guinea pig dies in less than 2 months, weigh and autopsy.
2. If the pig does not die in 2 months, weigh, kill, and autopsy.
3. See positive autopsy findings under pus (I).

III. Body Fluids (Pleural, Ascitic, or Spinal Fluid).

A. *Inoculation.*

1. Place in a sterile centrifuge tube and centrifuge at a high rate of speed for 45 minut
2. Pipette 1 cc. from the very top of the fl and pour off the supernatant fluid.
3. Add the fluid in the pipette to the sedime in the tube and mix.
4. Make 2 smears of the sediment; stain 1 wi Loeffler's methylene blue solution to see i bacteria are present and stain the other sme by the Ziehl-Neelsen method for acid-fa organisms.
5. If no bacteria are found with the methylene blue stain, inject part of the sediment subcutaneously into the groin of a guinea pig weighing about 300 gm. and the remainder intraperitoneally.
6. If there are many organisms present, digest with 5% oxalic acid as described under urine culture for tubercle bacilli on page 178 and then inject subcutaneously into the groin of a guinea pig.

B. *Autopsy.*

1. If the guinea pig dies in less than 2 months, weigh and autopsy.
2. If the pig does not die in 2 months, weigh, kill, and autopsy.
3. See positive autopsy findings under pus (I).

IV. Sputum.

A. *Digestion:* Use one of the methods described in the Section on Sputum on page 149.

B. *Inject sediment* subcutaneously into the groin of a guinea pig weighing about 300 gm.

1. If the guinea pig dies in less than 2 months, weigh and autopsy.
2. If the pig does not die in 2 months, weigh, kill, and autopsy.
3. See positive autopsy findings under pus (I).

V. Feces.

A. *Digestion:* Use the method described under tubercle bacilli in Section on Feces on page 128.

B. *Inject sediment* subcutaneously into the groin of a guinea pig weighing about 300 gm.

1. If the guinea pig dies in less than 2 months, weigh and autopsy.
2. If the pig does not die in 2 months, weigh, kill, and autopsy.
3. See positive autopsy findings under pus (I).

VI. Gastric Washing.

A. *Inoculation.*

1. Obtain gastric washings as described in Section on Gastric and Duodenal Contents and centrifuge at a high rate of speed for 45 minutes.

ve any surface scum or mucus which has
ot settled to the bottom of the tube by
ther removing it with a pipette or holding
back with an inoculating needle while pour-
g off the supernatant fluid.

dd the scum or mucus to the sediment and
igest with an equal volume of 2% NaOH
y placing in the 37°C. incubator for 30
inutes, shaking occasionally.

eutralize by adding 5% HCl until a green
olor appears using bromthymol blue as the
ndicator. Add 1 or 2 more drops of acid
o obtain a fine precipitate.

dd sterile 0.85% NaCl solution to fill the
ube.

entrifuge again at a high rate of speed for
5 minutes and pour off the supernatant fluid.

dd 2 cc. of sterile 0.85% NaCl solution,
nix with the sediment, and inject subcutane-
usly into the groin of a guinea pig weighing
bout 300 gm.

utopsy.

f the guinea pig dies in less than 2 months,
veigh and autopsy.

f the pig does not die in 2 months, weigh,
ill, and autopsy.

ee positive autopsy findings under pus (I).

VII. Tissue.

Preparation—grind in same manner as for culture, see page 184.

Inject 1 cc. of the suspension subcutaneously into the groin of a guinea pig and 1 cc. intraperitoneally.

If the guinea pig dies in less than 2 months, weigh and autopsy.

If the pig does not die in 2 months, weigh, kill, and autopsy.

See positive autopsy findings under pus (I).

Diphtheria Virulence Test

Principle: The same animal is used for the "test" and "control" injections. A suspension of the culture to be tested is injected intracutaneously into a nonimmune animal; 4 to 5 hours later, diphtheria antitoxin is given intraperitoneally or intravenously, and the same suspension again injected as a control into a different area of the skin of the same animal. The characteristic reaction of the skin produced by virulent diphtheria bacilli or toxin is not altered by the antitoxin because the damage done, during the time interval when the animal was not immune, is not repaired. The animal is rendered immune by the antitoxin, and the tissues are protected against a subsequent (control) injection of virulent diphtheria bacilli.

II. Materials Required.

A. *Animal.*

1. A white guinea pig weighing between 300 and 400 grams is used for the test.
2. Clip the hair on the back and flanks of the animal with an electric clipper.
3. Mark off squares approximately 4 cm. with an indelible pencil by drawing a line along the middle of the back of the animal and subsequent lines parallel and at right angles to it.
4. Do not use areas having pigmented skin, or areas anterior to the shoulder girdle, or over the sacrum.

B. *Suspension of Culture.*

1. Make a heavy, uniform suspension of the suspected organisms by adding 2 or 3 cc. of meat infusion broth to an 18 hour pure culture on Loeffler's serum medium.
2. The suspension should be the same density as the No. 3 tube of McFarland's nephelometer.
3. Use a tuberculin syringe and a 26 gauge needle for the injections.

III. Test Proper.

A. *Injection of Guinea Pig.*

1. Inject 0.1 cc. of the suspension of suspected organisms intracutaneously in one of the marked areas of the guinea pig. (The suspension may be kept in the syringe and stored in the refrigerator until the next injection.)
2. Four hours later inject 500 units of diphtheria antitoxin intraperitoneally.
3. One-half hour after the injection of the antitoxin, again inject 0.1 cc. of the suspension of organisms intracutaneously in a fresh area.
4. A positive control (known virulent, toxigenic strain of diphtheria) should also be injected before and after antitoxin.

B. *Reading Results.*

1. Read 48 hours after the inoculations.
2. The positive control (known virulent diphtheria organisms) injected before the antitoxin should give a reaction consisting of a central necrotic area 2 to 5 mm. in diameter, surrounded by a zone of redness 10 to 15 mm. in diameter. A central hemorrhagic area is usually present.
3. The positive control injection after the antitoxin usually exhibits a small pinkish papule about 5 mm. in diameter.
4. If the unknown organism gives a reaction similar to that of the known positive control, it is a virulent (toxigenic) organism.
5. An experienced technician need not use a positive control; however, if a field (not pure) culture is injected a positive control must be used.

Darkfield Examination

I. **Principle:** Darkfield illumination consists in blocking out the central rays of light and directing the peripheral rays against the microscopic object on the slide. Only those rays which strike the object are reflected upward and pass into the objective. The object appears bright upon a black background.

II. Apparatus Necessary.

A. ***Darkfield Condenser.***

1. It is a paraboloid condenser and is interchangeable with the regular substage Abbe condenser.
2. Remove the Abbe condenser from its substage adjusting sleeve and fasten the darkfield condenser in its place.
3. The upper lens surface must be in the plane of the upper surface of the stage or a little higher to assure close contact with the under surface of the glass slide.
4. The diaphragm of the condenser should be wide open.

B. ***Funnel Stop.***

1. The regular oil immersion lens has too great a numerical aperture for darkfield examination.
2. Insert a funnel stop, apex down, in the oil immersion lens by unscrewing the threaded end.
3. Screw the threaded end back in place and screw the lens back in the nose piece of the microscope.
4. This is not necessary if one has a special oil immersion lens with a numerical aperture of 0.80.

C. ***Lamp.***

1. A 6 volt ribbon filament bulb lamp (large microscope lamp) or a carbon arc lamp is placed about 12 inches in front of the microscope.
2. The beam of light should nearly fill the plane mirror.
3. When a carbon arc lamp is used, it is desirable to interpose a piece of ground glass between the light and mirror after the condenser has been focused.

III. Method.

A. ***Adjusting Microscope.***

1. Set up the darkfield microscope (see apparatus necessary) in a darkened room.
2. Adjust the plane mirror so that the dim reflected light spot from the mirror is reflected back into the light source from the lamp.
3. The low power objective is focused on the upper surface of the condenser before placing the preparation on the stage.
4. When some light passes into the condenser even though the illumination is not uniform a small circle will be seen scratched upon the surface of the top lens. The circle is in the center of the lens and it must be placed in the exact center of the field by means of the centering screws on the condenser.

B. ***Preparation of Smear to be Examined.***

1. Lesions should be thoroughly cleaned with gauze soaked in sterile saline to remove the surface crust, detritus, and contaminating organisms.
2. Dry and collect serum from the depth of the lesion by means of a fine capillary pipette.
3. Place a small drop of the material on a scrupulously clean new slide (1.45 to 1.55 mm thick) and cover with a clean No. 1 cover glass. Avoid air bubbles.
4. The emulsion must be thin as too many particles will cause too much scattering of light and diminish the sharpness of the field. The correct thickness of the smear is obtained when the material just reaches to the edges of the cover glass when slight pressure is put on the cover glass.
5. There must not be too much debris, pus, or blood.

C. ***Examination of Smear.***

1. Lower the substage and place a drop of cedar oil, free from bubbles, on the upper surface of the condenser.
2. Place the slide preparation on the stage and center the specimen.
3. Raise the substage until the oil is spread by contact with the slide, filling the space between the slide and condenser.
4. Examine the smear with the low power objective. If the condenser is in the proper position, a circle of light will appear in the center of the field.
    a. If the condenser is too high or too low, a ring of light with a dark center will be seen.
    b. Raise or lower the condenser until a circle is obtained.
5. Place a drop of oil on the smear and examine with the oil immersion lens.
6. The background should be dark and the scattered bodies in the field should be brilliantly illuminated. Slight adjustment of the height of the condenser, position of the mirror, and closing the diaphragm may improve the brilliance of the illuminated bodies.
7. *Treponema pallidum.*
    a. Cylindrical, flexible, delicate body, 0.25

to 0.3 wide by 6 to 14 microns long.

Ends are pointed, sometimes prolonged in delicate terminal filaments.

Body coiled in 8 to 14 regular rigid sharp spirals with a spiral amplitude of about 1 micron and spiral depth of 0.5 to 1 micron.

May show one or more slight, undulating curves.

Motility chiefly rotational with slow, graceful undulations.

Treponema pallidum never shows double contours under the darkfield.

he following nonpathogenic Treponema and orrelia may be confused with pallidum in aterial from lesions on the genitalia.

Treponema genitalis.
  1) Cylindrical body 0.25 to 0.3 by 3 to 14 microns, average length 7 to 10 microns.
  2) Body has 7 to 10 spirals with an amplitude of about 1 micron and a depth of 0.2 to 0.5 micron.

Treponema calligyrum.
  1) Cylindrical body 0.35 to 0.4 by 6 to 14 microns, average length 9 to 12 microns.
  2) Ends are sharp points with delicate projections.
  3) Body has 5 to 8 spirals with an amplitude of about 1.6 microns and depth about 1.0 to 1.5 microns.

Borrelia refringens.
  1) Body is 0.5 to 0.75 by 6 to 20 microns.
  2) The number of curves differs; their amplitude is 3 microns.
  3) The ends are pointed with curved, flagella-like projections.

Borrelia phagedenis.
  1) Body is 0.7 to 0.8 by 10 to 15 microns showing 1 or 2 curves.
  2) The extremities are fairly pointed.

9. The following nonpathogenic Treponema may be confused with pallidum in smears from mucous patches of the mouth.
   a. *Treponema microdentium.*
      1) Length about 8 microns and less than 0.25 micron in thickness in middle, tapering toward each extremity which is pointed.
      2) Average of 14 spirals.
      3) Motility is characterized by rapid rotation and its flexions, which are rarely present, are stiff.
   b. *Treponema macrodentium.*
      1) Body is 0.7 to 1.0 by 3 to 8 microns with abruptly tapering extremities.
      2) Spirals vary from 2 to 8.
   c. *Treponema mucosum.*
      1) Body is 0.25 to 0.3 by 8 to 12 microns; both extremities are sharply pointed and often possess a minute curved projection, 8 to 10 microns long.
      2) Spirals vary from 6 to 8.

IV. Sources of Error in Darkfield Examination.

1. Insufficient illumination.
2. Condenser out of focus or decentered.
3. Glass slide or cover glass too thick.
4. Preparation too dense.
5. Failure to use funnel stop of correct numerical aperture.
6. Failure to close iris diaphragm.
7. Inclusion of air bubbles in the preparation or in the oil above or below the slide.

Mycology*

I. General Considerations.

A. ***Fungi are important infectious agents*** because of:

1. The direct effects of infection with these organisms, as in actinomycosis, sporotrichosis, various dermatomycoses, etc.
2. Allergic phenomena resulting from the development of hypersensitivity to their products, as in some forms of asthma.
3. Combined effects of infection and hypersensitization, as in athlete's foot.

B. ***Fungi are an important source of antibiotics,*** such as penicillin.

II. Classification.

A. ***Pseudomycetes*** **(False Fungi).**

1. *Schizomycetes*—bacteria, Actinomyces, and Nocardia.
2. *Myxomycetes*—slime molds (nonpathogenic).

B. ***Eumycetes (True Fungi)*** are identified and classified by the type of colony produced, the presence or absence of mycelium, the type of mycelium, the character of the spores, and the method of spore development.

1. *Phycomycetes*—characterized by nonseptate mycelium, asexual spores (sporangiospores) and sexual spores (zygospores and oospores), i.e., Mucor.
2. *Ascomycetes*—characterized by septate mycelium, sexual spores (ascospores), and asexual spores (conidia), i.e., Saccharomyces (yeasts), Aspergillus, Penicillium, Coccidioides, and Dermatophytes.
3. *Basidiomycetes* — characterized by septate mycelium and sexual spores (basidiospores), i.e., rusts, smuts, and mushrooms.
4. *Fungi Imperfecti*—characterized by septate mycelium and asexual spores (thallospores and conidia), i.e., Candida, Cryptococcus, Sporotrichum, Hormodendrum, and Malesseзia.

III. Direct Smears for Fungi.

A. ***Dried Smear.***

1. Place a loopful of material on a slide, dry, fix, and stain either by Gram's method or with methylene blue solution.
2. Look for yeast cells and mycelia.

B. ***Moist Preparation.***

1. Place a loopful of material on a slide and while still wet add a drop of 10% KOH or NaOH and then cover with a cover glass.
2. Warm gently over a low flame to clear and examine with the high dry objective of the microscope.
3. The alkali dissolves the pus and debris in the material making the mycelia more distinct.
4. A moist preparation, without alkali, with the edge of the cover glass rimmed with vaseline may be kept at room temperature for several days for the observation of mycelial growth.

IV. Cultures for Fungi.

A. ***Follow directions*** under the description of the particular fungus suspected.

B. ***Routine Cultures.***

1. Inoculate a blood agar plate, 2 Sabouraud's glucose agar slants, 2 glucose agar slants, and 1 tube of glucose broth. When inoculating the Sabouraud's agar slants use a straight inoculating wire so that the material may be pushed into the medium.
2. Incubate all media at 37°C., except 1 Sabouraud's agar slant and 1 dextrose agar slant; these are left at room temperature in the dark.
3. Growth usually appears in from 2 to 7 days, but cultures should be kept 3 weeks before reporting negative.

C. ***Littman's Medium.***

1. This is an excellent medium to use when materials such as sputum and feces, which contain many organisms, are to be cultured.
2. Formula:

Glucose	5.0 gm.
Peptone (Bacto-granular)	5.0 gm.
Oxgall (Bacto-dehydrated)	7.5 gm.
Agar	10.0 gm.
Distilled water	500.0 cc.
Crystal violet solution	0.1 cc.

3. Distribute in flasks in 100 cc. portions and autoclave at 10-12 lbs. pressure for 15 minutes (115-117°C.). Do not heat over this temperature or pressure because of the heat instability of oxgall.
4. Store in the refrigerator.

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*This material has been outlined from the "Manual of Clinical Mycology" by N. F. Conant, D. S. Martin, D. T. Smith, R. D. Baker, and J. L. Callaway, published by W. B. Saunders Company.

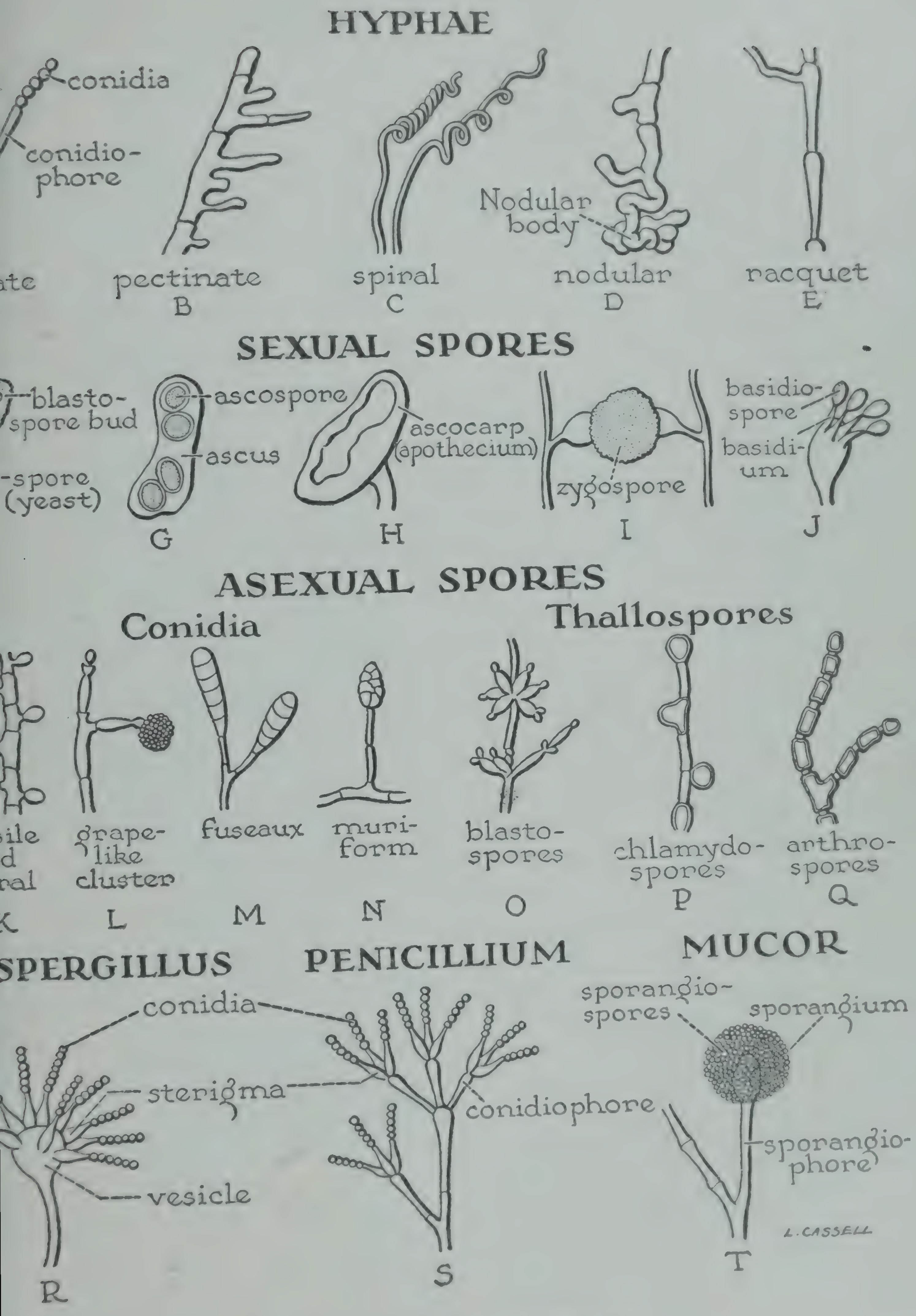

HYPHAE
conidia
conidiophore
ate
pectinate
B
spiral
C
Nodular body
nodular
D
racquet
E
SEXUAL SPORES
blasto-spore bud
-spore (yeast)
ascospore
ascus
G
ascocarp (apothecium)
H
zygospore
I
basidiospore
basidium
J
ASEXUAL SPORES
Conidia
Thallospores
sile
d
ral
K
grape-like cluster
L
fuseaux
M
muriform
N
blastospores
O
chlamydospores
P
arthrospores
Q
SPERGILLUS
PENICILLIUM
MUCOR
conidia
sterigma
vesicle
R
conidiophore
S
sporangiospores
sporangium
sporangiophore
T
L. CASSELL

Fig. 22. Illustrations of Terms Applied to Fungi.

5. When ready to use, melt agar, cool to 46°C., and add streptomycin sulfate dissolved in sterile 0.85% NaCl solution so that 1 cc. of the solution when added to 100 cc. of medium will give a final concentration of 30 micrograms per cc. of agar.
6. Distribute 27 to 30 cc. in a Petri dish, leave at room temperature 6 to 8 hours, then store in the refrigerator.
7. Streak plates with a generous quantity of sputum or fecal suspension in saline, and incubate at room temperature.
8. *Crystal Violet Solution.*

Crystal violet (91% dye content)	1.25 gm.
Ethyl alcohol (95%)	25.0 cc.

Keep in a tightly stoppered bottle.

D. *Henrici's Slide Culture Method.*

1. Prepare a glass slide and cover glass as follows:
   a. Wash a glass slide and No. 1 cover glass (22 x 40 mm.) with alcohol and pass through a flame.
   b. Melt sealing wax and place a small amount along the two ends of the cover glass.
2. Inoculate a tube of Sabouraud's agar (melted and cooled to 45°C.) with a portion of the fungus culture and mix by rotating between the palms of the hands.
3. With an inoculating needle spread a thin layer of the inoculated agar over an area on the prepared slide the size of the cover glass.
4. Flame the cover glass to remelt the sealing wax and drop on the glass slide over the agar to form a chamber about 1 mm. in depth, sealed at the ends.
5. Support the slide in a covered dish containing water and incubate at room temperature.
6. Examine daily with the low power objective of the microscope for hyphae with spores.
7. The slide should be handled carefully, as slight jarring will dislodge the spores from the hyphae.

E. *Appearance of Colonies on Glucose Agar.*

1. *Yeast Colony.*
   a. It appears small, dry, soft, chalky white, and opaque, somewhat resembling a colony of staphylococcus.
   b. In old cultures they become quite rough and wrinkled.
2. *Monilia Colony.*
   a. It appears like a yeast colony on the surface but forms pseudomycelia in the substrate and is therefore called a yeast-like colony.
   b. It is best studied in a moist preparation or by Henrici's slide culture method.
3. *Fungus Colony.*
   a. It appears fuzzy, is very tough and leathery in consistency because it is composed of feet-like mycelia, is deeply embedded in the agar, and is called a filamentous colony.
   b. Growth spreads over the surface of the medium and is usually white, gray, black or varying shades of green.

F. *Smears of Growth on Cultures.*

1. Make a smear of any growth and stain by Gram's method.
2. Fungi are best studied in a moist preparation.
   a. If the fungus produces a light, cottony growth well above the surface of the medium, a small portion of this growth may be placed in a drop of 0.85% NaCl solution on a slide and covered with a cover glass.
   b. Sometimes it is necessary to cut out a triangular segment from the edge of a colony with a strong straight inoculating wire or dissecting needle.
      1) A portion of the center of the colony should be included in order that well-developed fruiting bodies and spores may be present.
      2) It may be necessary to remove some agar.
      3) Place the material in a drop of 0.85% NaCl solution on a slide and cover with a cover glass.
      4) Carefully press the preparation until the material is crushed to a thin film.
   c. Examine with the high dry objective of the microscope.

V. Terms Applied to Fungi.

A. *Hyphae* are long filaments which develop from germinating spores; some may branch.

1. A *septate hypha* is one which is divided into a chain of cells by cross walls or septa (Fig. 22, A).
2. A *nonseptate hypha* is one without septations.
3. A *pectinate hypha* is one with lateral projections resembling a comb (Fig. 22, B).
4. A *spiral* is a corkscrew-like turn of a terminal hypha (Fig. 22, C).
5. A *nodular body* is an enlargement consisting of closely twisted hyphae which may be formed by side branches turning around the main stem or by different filaments (Fig. 22, D).
6. A *pseudohypha* is a filament formed by buds which elongate and do not become detached from the parent cell and by repeated budding form a branching network made up of long

ins of the attached cells.

conidiophore is a specialized hypha which oduces conidia, i.e., spores (Fig. 22, A).

In *Aspergillus*, the end of the conidiophore is swollen (a vesicle) and from this several flask-shaped structures called sterigma are produced (Fig. 22, R).

In *Penicillium*, sterigma are produced without a vesicle; the conidiophore sometimes branches to give a brush-like appearance (Fig 22, S).

ycelium is a matted growth of hyphae.

egetative mycelium is one which penetrates e substrate and absorbs food for growth.

erial mycelium is one which projects above e surface of the substrate and produces ores.

eudomycelium is one which is made up of eudohyphae.

acquet mycelium is formed by hyphal cells hich are swollen at one end and small at e other end and arranged so that the large d is attached to the small end of the adcent cell (Fig. 22, E).

exual spores are formed by primary nuclear sion.

scospores occur in groups in a sac which called an ascus (Fig. 22, G).

n *ascocarp* is a fruiting body containing veral asci; it may be spherical or flaskaped (perithecium) or open and saucer or up-shaped (apothecium). See Fig. 22, H.

zygospore is a large thick-walled body ormed by the fusion of two similar spores f approximating hyphae (Fig. 22, I).

n *oospore* develops when a special female tructure on a hypha is fertilized by the nuleus of a male structure developed close by; he structure which contains the oospore is alled the oosphere.

A *basidiospore* develops from the end of a lub-shaped structure called a basidium (Fig. 2, J).

Asexual spores are formed by budding without fusion of the nuclei.

Sporangiospores occur in groups in a swollen structure or sporangium on the end of a hypha which is called a sporangiophore. A typical example is Mucor (Fig. 22, T).

Conidia usually develop from specialized hyphae called conidiophores and are freed by abstriction at the point of attachment (Fig. 22, A). They sometimes develop directly on the side of a hypha without conidiophores and are said to be sessile and lateral (Fig. 22, K). They may produce clusters (en grappe) on very short hyphal branches (Fig. 22, L), or they may develop on a short conidiophore (pedicle) and are said to be pedunculated (see conidiophore under hyphae).

a. *Microconidia* are small single-celled conidia and may be round (spherical), egg-shaped (elliptical or oval), pear-shaped (pyriform), or club-shaped (clavate).

b. *Macroconidia* are large, usually multicelled conidia.
   1) They may be divided by transverse septations and appear spindle-shaped (fusiform) or club-shaped (clavate) and are called *fuseaux* (Fig. 22, M).
   2) They may be divided by both transverse and longitudinal septations and are called *muriform* (Fig. 22, N).

3. *Thallospores* are formed by mycelia.
   a. *Blastospores* are formed by budding processes from the hyphae; also the buds produced by yeast cells in reproduction are called blastospores (Fig. 22, F and O).
   b. *Chlamydospores* are round thick-walled resting spores (Fig. 22, P).
      1) They may be formed from the terminal cells of pseudohyphae.
      2) They are formed in filamentous colonies by cells of the hyphae concentrating their protoplasm and becoming larger than the diameter of the hyphae.
      3) Those formed in the hypha are called terminal and those on the side of the hypha are called lateral chlamydospores.
   c. *Arthrospores* are formed by segmentation of the hyphae, which results in the cutting off of rectangular, somewhat thick-walled cells (Fig. 22, Q).

VI. Actinomycosis (Actinomycetes and Nocardia).

A. *Direct Examination.*

1. Examine for tiny yellow granules, "sulfur granules," in pus from sinuses, in material curetted from the walls of the lesion, in sputum, or in spinal fluid; see Fig. 23, A.
   a. Place a granule on a slide, add a drop of 10% KOH, and crush with a cover glass.
      1) Actinomyces granules appear as lobulated bodies composed of delicate, branching, intertwined filaments (1 micron in diameter), the ends of which frequently are surrounded by a gelatinous sheath giving a club-shaped appearance to the ends of the filaments.
      2) The clubs form a palisaded arrangement around the central body.

   b. Crush a granule on a slide and stain by Gram's method; the filaments are gram-positive.
   c. Crush another granule on a slide and stain by Ziehl-Neelsen method; A. bovis and N. madurae are not acid-fast, while N. asteroides and N. gypsoides are acid-fast.
2. Organized granules may not be present in spinal fluid or sputum, only short gram-positive or acid-fast branching elements.

B. *Cultures.*

1. Streak 2 Sabouraud's glucose agar slants and incubate one at 37°C. and one at room temperature.
2. Inoculate a deep tube of beef or veal infusion broth (pH 7.6-7.8) and a tube of thioglycollate glucose broth; incubate at 37°C.
3. Inoculate a veal or beef infusion glucose agar shake tube and incubate at 37°C.

C. *Identification.*

1. *Actinomyces bovis* (anaerobic).
   a. It does not grow on Sabouraud's agar.
   b. Growth occurs in the bottom of the beef infusion or thioglycollate broth tube as small, fuzzy, white colonies which are easily broken up by shaking.
   c. In the shake tube growth appears in 3 to 4 days as small, white, fuzzy, or lobulated colonies about 1 cm. below the surface of the agar; larger colonies are found deeper in the agar.
   d. Place a colony in a drop of 0.85% NaCl solution on a slide and crush with a cover glass; A. bovis appears as a tangled mass of delicate branching filaments.
   e. Make a smear from the culture and stain by Gram's method. The filaments are gram-positive and frequently resemble diphtheroids.
2. *Nocardia* (aerobic).
   a. Appearance of growth on Sabouraud's agar.
      1) N. asteroides colonies are glabrous, irregularly folded, and vary in color from pale yellow to deep orange.
      2) N. gypsoides colonies are similar but chalky white.
      3) N. madurae colonies are glabrous, waxy, wrinkled, cream-colored, later becoming pink to red.
   b. Appearance in moist preparations and stained smears is similar to that of A. bovis.
      1) All species are gram-positive with a Gram stain.
      2) With a Ziehl-Neelsen stain, N. asteroides and N. gypsoides are acid-fast, while N. madurae is not acid-fast.

D. *Animal Inoculation.*

1. Sputum can be inoculated subcutaneou guinea pigs if acid-fast Nocardia is susp
2. The concentration methods for tubercle cilli kill the Nocardia and must not be

E. *Interpretation.*

1. A. bovis, N. asteroides, and N. gypsoide the etiologic agents of actinomycosis.
2. N. madurae is one of the causative ag maduromycosis, "Madura foot"; see Fig. 2
3. For N. tenuis see trichomycosis axillaris, p 219.
4. For N. minutissima see erythrasma, page

VII. Blastomycosis (Blastomyces).

A. *Direct Examination.*

1. Examine scrapings of tissue, pus from unde mined borders of a lesion, pus from subcutan ous abscesses, and sputum, urine, or spin fluid in systemic infections.
2. Make a moist preparation of the materi using a drop of 10% KOH.
   a. B. dermatitidis appears as single or buddin spherical cells, 8 to 15 microns in diamete with a thick refractive wall giving a "dou ble-contoured" appearance; see Fig. 23,
   b. B. brasiliensis appears as single or multip budding, thick-walled, spherical cells, 1 to 60 microns in diameter.
   c. If no budding is seen in the direct smea rim the cover glass with vaseline and loo for budding in 24 to 48 hours.
   d. Mycelial filaments are not present in th tissues in either type of Blastomyces.

B. *Culture.*

1. Inoculate two Sabouraud's glucose agar slan and incubate one at 37°C. and one at roo temperature.
2. Inoculate a blood agar or beef infusion gl cose agar slant and incubate at 37°C.
3. The organisms grow very slowly and coloni may not appear for 3 to 4 weeks.

C. *Identification.*

1. *B. dermatitidis.*
   a. On all media incubated at 37°C., the co onies appear wrinkled and waxy, whi smears of the growth show budding yeas like cells identical with those found the pus from lesions.
   b. On Sabouraud's glucose agar at room te perature, the mycelial phase of the fung predominates.
      1) A white, cottony, aerial growth a pears which becomes tan to brown wi age.
      2) Smears show septated mycelia wi oval to round conidia attached ne

septations and round to pyriform conidia borne on lateral sterigmata. In old cultures many chlamydospores are developed. See Fig. 23, C.

brasiliensis.

On all media incubated at 37°C., the colonies appear smooth to cerebriform, and yeast-like, while smears of the growth show single and multiple budding cells identical with those found in the infected material.

On Sabouraud's glucose agar at room temperature, the mycelial phase predominates.

1) The colonies are heaped up, membranous, and wrinkled with a short nap of white mycelium which becomes brown with age. A few strains remain glabrous and wrinkled.
2) Smears show a few sessile, oval to round conidia attached near septations of the mycelium.

nimal Inoculation.

fected material or a saline suspension of a lture may be injected intraperitoneally into ice or intratesticularly into guinea pigs.

bscesses may appear in 3 or 4 weeks in the er, spleen, lungs, lymph nodes, and testicles.

he organism can be found in the abscesses.

terpretation.

dermatitidis is the cause of North American astomycosis.

brasiliensis is the etiologic agent of South merican blastomycosis.

III. Coccidioidomycosis (Coccidioides immitis).

irect Examination.

xamine sputum, gastric contents, pleural uid, pus from subcutaneous abscesses, and udates of cutaneous lesions in moist preparaon.

dd 10% KOH to scrapings from lesions hen making moist preparations.

. immitis appears as a nonbudding, spherical, ick-walled structure, 20 to 80 microns in ameter and filled with numerous endospores; e Fig. 23, E.

here are no mycelial filaments in direct nears.

ulture and Identification.

noculate Sabouraud's glucose agar slants and cubate at room temperature.

he colony at first is moist and membranous d then develops a white cottony appearance hich becomes tan to brown with age.

3. Wet preparations from cultures show branching, septate hyphae which break up into numerous thick-walled, rectangular, ellipsoidal. or spherical arthrospores.
4. These cultures are extremely dangerous to have in a laboratory and great care must be taken in transferring the cultures.

C. *Animal Inoculation.*

1. Infected material can be inoculated into the testes of guinea pigs which develop a severe orchitis in 7 to 10 days.
2. Guinea pigs are not nearly so susceptible to B. dermatitidis, the organism usually to be distinguished from C. immitis.

D. *Skin Test.*

1. Inject 0.1 cc. of a 1-100 dilution of standardized coccidioidin intracutaneously in the forearm and read in 48 hours.
2. An area of erythema or induration of 0.5 cm. or larger is considered a positive test.
3. If negative, inject a 1-10 dilution.
4. A positive test indicates that an infection has occurred at some time during the patient's life.
5. The test may be negative if the patient is anergic which is not unusual when the lesions become widespread in the terminal stages of the infection.

E. *Interpretation.*

1. C. immitis is the etiologic agent of coccidioidomycosis which may be in one of two forms.
    a. Primary coccidioidomycosis which is usually an acute but benign, self-limited respiratory disease.
    b. Progressive coccidioidomycosis which is a chronic, malignant and disseminated disease involving cutaneous, subcutaneous, visceral, and osseous tissue.
2. The disease is endemic in southwestern United States with the greatest focus in the San Joaquin Valley in California.

IX. Torula (Cryptococcus neoformans or Torula histolytica).

A. *Direct Examination.*

1. Examine exudate from lesions or sputum by moist preparation.
2. Spinal fluid should be centrifuged and the sediment placed on a slide and examined before drying.
3. The light coming through the microscope must be subdued in order to see the capsule of the organism which is transparent and difficult to see.
4. A drop of dilute India ink may be added to

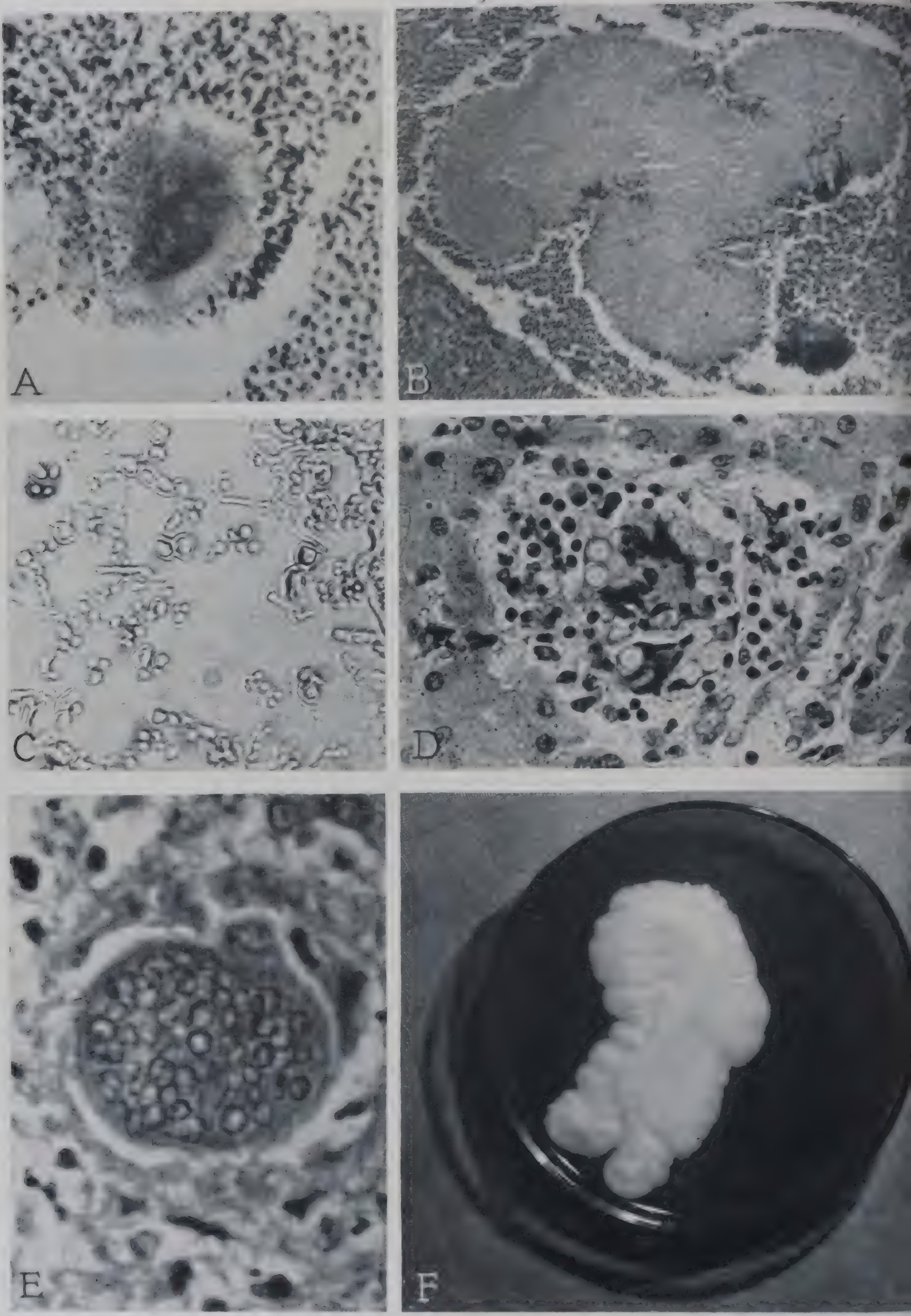


Fig. 23. A. "Sulfur granule," Actinomyces bovis. B. Granules from a lesion of Maduromycosis. C. Blastomyces de titidis in a moist preparation made from a culture. D. Blastomyces dermatitidis in tissue. E. Coccidioides in tissue. F. Colony of Cryptococcus neoformans (Torula histolytica). (Courtesy of Dr. A. C. Curtis, Profess Dermatology, University of Michigan, Ann Arbor.)

ncellular material (spinal fluid) to make
 capsule stand out, but it must be exam-
d immediately to avoid artifacts as a re-
t of drying.

e organism is ovoid to spherical, single-
dding, thick-walled, yeast-like, 5 to 20 mi-
ns in diameter and surrounded by a wide,
htly refractive gelatinous capsule.

ltures and Identifications.

oculate a blood agar plate, beef infusion
ucose agar slant, and Sabouraud's glucose
nt and incubate at 37°C.

oculate another Sabouraud's glucose agar
nt and keep at room temperature.

t room temperature the colonies on Sabou-
ud's agar slant are at first white, wrinkled,
d granular, later becoming moist, slimy,
ucoid, and cream to brownish in color; see
g. 23, F.

nears of the early growth show budding
lls and organisms with short germ tubes;
hile the later growth (mucoid) shows only
udding cells and capsules which can be dem-
nstrated by India ink.

olonies on Sabouraud's agar at 37°C. appear
ke the mucoid growth at room temperature
nd the microscopic appearance is similar.

nimal Inoculation.

nfected material or saline suspensions of a
ulture may be injected intraperitoneally into
nice.

The organism may be found in gelatinous
nasses in the mesentery, in peritoneal lymph
odes, or in brain tissue after 3 or 4 weeks.

nterpretation.

. neoformans is the cause of cryptococcosis
r torulosis.

t may cause cutaneous, subcutaneous, or
landular lesions, pulmonary lesions or invade
he central nervous system.

X. Histoplasmosis (Histoplasma capsulatum).

irect Examination.

Make thick and thin smears of peripheral
lood in the same manner as for malaria; see
age 68.

Centrifuge citrated blood and make smears
of the buffy layer and stain by Wright's or
Giemsa's method.

Obtain bone marrow by a sternal puncture
and make a smear and stain by Wright's or
Giemsa's method.

Make sections of a biopsy of the enlarged
lymph nodes.

5. Examine smears and tissue sections for Histoplasma capsulatum which are small (1 to 5 microns) oval bodies in the mononuclear cells; occasionally budding organisms can be seen; see Fig. 24, B.

B. *Cultures and Identification.*

1. Culture bone marrow, sputum, lymph nodes, or material from ulcerations in glucose broth or on blood agar and Sabouraud's glucose agar.
    a. Incubate all cultures except Sabouraud's agar at 37°C.; this is incubated at room temperature.
    b. All cultures must be kept one month.
2. Make a routine blood culture.
3. Small flocculent masses appear in positive broth cultures, which upon microscopic examination appear as branching septate hyphae without spores.
4. Growth appears on the blood agar as moist, scattered, dull-white colonies which on microscopic examination consist of small budding cells and short fragments of mycelia.
5. Growth is slow on Sabouraud's agar and appears as white, cottony, aerial mycelia which later turn buff to brown in color; see Fig. 24, A.
    a. Microscopic examination shows branching septate hyphae bearing small, round to pyriform, smooth spores on short lateral branches or sessile on the sides of the hyphae and appear like cultures of Blastomyces dermatitidis.
    b. Later the characteristic round to pyriform, tuberculate chlamydospores develop.

C. *Animal Inoculation.*

1. Infected material or a saline suspension of a culture may be inoculated intraperitoneally into guinea pigs or mice.
2. Tubercle-like lesions appear in the visceral organs in which H. capsulatum may be found in the macrophages.

D. *Skin Test.*

1. A skin test done in the same manner as the tuberculin test using histoplasmin has been used experimentally.
2. Results so far do not justify its use in diagnosis.

E. *Interpretation.*

1. H. capsulatum is the cause of histoplasmosis which is characterized by leukopenia, anemia, irregular pyrexia, and emaciation.
2. There is frequently lymphadenopathy, splenomegaly, hepatomegaly, and ulcerations of the intestine and the naso-oral-pharyngeal cavities.

XI. Moniliasis (Candida albicans).

A. ***Direct Examination.***

1. Mount skin and nail scrapings on a slide in a drop of 10 or 20% KOH, add a cover glass, and heat preparation gently over a low flame for immediate clearing.
2. Crush a loopful of sputum or material from mucous patches in the mouth or vagina on a slide to a thin film under a cover glass and examine immediately. Also make smears and stain by Gram's method.
3. Species of Candida appear as small, oval, budding, thin-walled, yeast-like cells, 2 to 4 microns in size.
4. Occasionally mycelial threads are seen with budding cells attached to the hyphae at the points of constriction; see Fig. 24, D.

B. ***Cultures and Identification.***

1. Inoculate 2 Sabouraud's glucose agar slants with scrapings or swabs of the lesions and incubate one at 37°C. and the other at room temperature.
2. Creamy, medium-sized, moist to dull colonies appear in 4 to 5 days, which have a distinct yeast-like odor; see Fig. 24, C.
3. For quick identification, pick organisms from the Sabouraud's slant with a straight wire and inoculate a thin corn meal agar plate by making a deep cut into the agar; incubate at room temperature for 48 hours.
    a. Examine through the bottom of the Petri dish by means of the low power objective of the microscope.
    b. C. albicans produces mycelium bearing ball-like clusters of budding cells and thick-walled round chlamydospores.
4. Fully identify (see Table 46) in the following manner:
    a. Transfer growth isolated on Sabouraud's agar slant to Sabouraud's glucose acid broth, incubate at 37°C. for 48 hours, and note the type of surface growth.
    b. Shake the broth culture to suspend the sedimented organisms and streak on a blood agar plate at pH 7.4; incubate at 37°C. for 10 days and note the type of colony.
    c. Transfer a well-isolated colony to a Sabouraud's agar slant and incubate at room temperature or at 37°C. for 24 or 48 hours.
    d. Subculture on a beef extract agar slant pH 7.4 for 2 or 3 generations.
    e. With a loopful of the last subculture, make a Henrici slide culture using corn meal agar and examine for mycelial growth.
    f. Inoculate 4 broth tubes containing 1% glucose, sucrose, lactose, and maltose respectively with a saline suspension last transplant of the fungus on the extract agar slant, using a pipette to the transfer.
    g. Observe after 48 hours and 1 week; Table 46 for sugar reactions.

C. ***Animal Inoculation.***

1. C. albicans is the only Candida pathog for animals.
2. A rabbit injected intravenously with 1 cc. a 1% saline suspension of a culture of albicans will die in 4 or 5 days with swolle kidneys showing numerous small white abscesses scattered throughout the cortex.
3. Intracutaneous injections into rabbits resul in abscess formation in 48 hours.

D. ***Interpretation.***

1. C. albicans is the only pathogenic Candida.
2. It produces oral, vaginal, cutaneous, bronchial and pulmonary moniliasis.

XII. Sporotrichosis (Sporotrichum schenckii).

A. ***Direct Examination.***

1. Make a smear of pus from the lesion and stain by Gram's method.
2. The organisms appear as small, gram-positive cigar-shaped bodies within pus cells or giant cells.
3. The organisms are found rarely in direc smears so diagnosis depends on cultures.

B. ***Cultures and Identification.***

1. Inoculate a blood agar plate and a Sabouraud' glucose agar slant with pus.
2. Incubate the plate at 37°C. and the Sabouraud's glucose agar slant at room temperature
3. On Sabouraud's agar small white colonies appear in 3 to 5 days; as growth increases they become moist, wrinkled, and membranous and vary in color from cream to black; see Fig 24, E.
4. Moist preparation of the growth shows delicate, branching, septate hyphae with latera conidia or groups of conidia on the end of short lateral branch.
5. The conidia are pyriform, ovoid to spherical but become round and thick-walled in ol cultures.

C. ***Animal Inoculation.***

1. Pus or a saline suspension of a culture ma be inoculated intraperitoneally into mal white rats.
2. The animals develop peritonitis and sever orchitis and smears of the lesions reveal numerous gram-positive, cigar-shaped, intracellular organisms.

Table 46. Differential Diagnosis of Species of Candida (From Martin et al., 1937)

	Pathogenic	Nonpathogenic					
	C. albicans	C. tropicalis	C. psuedotropicalis	C. krusei	C. parakrusei	C. stellatoidea	C. guilliermondi
Sabouraud's agar	Creamy growth.	Not characteristic.	Not characteristic.	Flat, dry.	Creamy.	Creamy.	Creamy growth.
Sabouraud's broth	No surface growth.	Narrow surface film with bubbles.	No surface growth.	Wide surface film.	No surface growth.	No surface growth.	No surface growth.
Blood agar	Medium-sized dull-gray colonies.	Large, gray colonies surrounded by mycelial fringe.	Colonies small, not characteristic.	Colonies small irregularly-shaped, flat or heaped.	Colonies small, brilliant white.	Colonies star-shaped.	Medium-sized dull-gray colonies.
Corn meal	Branched, tree-like mycelium with chlamydospores.	Mycelium well-developed, branched, bearing numerous blastospores, no chlamydospores.	Mycelium poorly developed, no chlamydospores.	"Crossed sticks" mycelium, no chlamydospores.	Mycelium well-developed, no chlamydospores.	Mycelium with large, ball-like clusters of blastospores.	Mycelium well-developed, no chlamydospores.
Glucose	AG	AG	AG	AG	AG	AG	–
Maltose	AG	AG	–	–	–	AG	–
Sucrose	A	AG	–	–	–	–	–
Lactose	–	–	AG	–	–	–	–

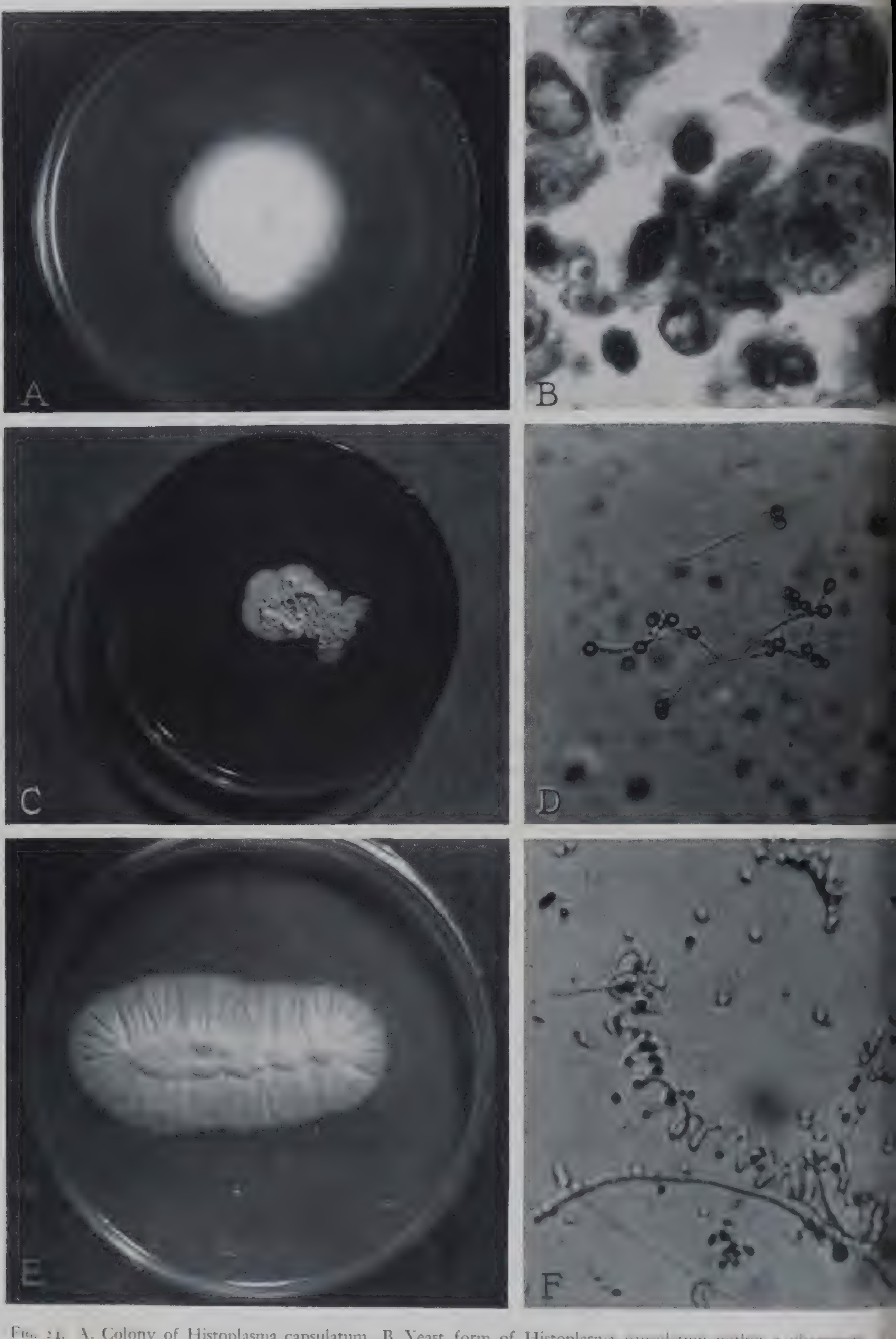


FIG. 24. A. Colony of Histoplasma capsulatum. B. Yeast form of Histoplasma capsulatum within a phagocyte. Colony of Candida albicans. D. Hyphae and spores of Candida albicans. E. Colony of Sporotrichum schenck F. Spiral hyphae of Trichophyton mentagrophytes in a culture mount. (Courtesy of Dr. A. C. Curtis, Profess of Dermatology, University of Michigan, Ann Arbor.)

nterpretation.

orotrichum schenckii is the cause of sporo- ichosis, a chronic infection characterized by dular lesions in lymph nodes, skin, or sub- taneous tissue which soften and break down form indolent ulcers.

he fungus is often found on plants and the nfection occurs most frequently in farmers, borers, and horticulturists.

XIII. Geotrichosis (Geotrichum).

irect Examination.

lake a moist preparation of pus, sputum, urulent or bloody bits of feces.

eotrichum appears as oblong or rectangular ells, 4 by 8 microns, with somewhat rounded nds or as large spherical cells, 4 to 10 mi- rons in diameter.

ultures.

Inoculate 2 Sabouraud's glucose agar plates with infected material; incubate one at room temperature and one at 37°C. for at least 2 weeks.

At 37°C. there is a small central colony growth on the surface of the agar surrounded by a marked, wide zone of mycelium growing down into the agar.

At room temperature the fungus grows at a moderately fast rate and develops a dry, mealy surface which is easily picked up with an inoculating loop.

Identification.

Make a moist preparation of some of the mycelial growth.

The hyphae segment into rectangular arthrospores which vary in size and roundness of their ends.

Many spherical cells, 4 to 12 microns in diameter, are segmented from the hyphae.

The rectangular cells usually germinate by a germ tube from one corner, which is very characteristic of Geotrichum.

The spherical cells may develop what appear to be buds which later elongate to become septate branching hyphae.

Interpretation.

Geotrichum produces lesions in the mouth, bronchi, lungs, and intestine.

Before this fungus is accepted as the causative agent in any given patient, it must be found on repeated examinations of direct smears as well as in cultures, because it is frequently found in normal persons.

XIV. Aspergillosis, Penicillosis, and Mucormycosis.

A. *Direct Examination.*

1. Make a moist preparation of pus or sputum.
2. Examine for the characteristic conidiophores of Aspergillus and Penicillium, and sporangiophores of Mucor; see Fig. 22, page 205.

B. *Cultures.*

1. All of these organisms grow well on Sabouraud's glucose agar at room temperature.
2. They are the most common laboratory contaminants; therefore, positive cultures must be interpreted with caution.

C. *Interpretation.*

1. Aspergillosis is characterized by inflammatory granulomatous lesions in the skin, external ear, nasal sinus, orbit, bronchi, or lungs and occasionally in the bones and meninges.
2. Penicillosis is characterized by a pulmonary lesion similar to an abscess and by otomycosis.
3. Mucormycosis has occurred in epidemics of paronychia in orange workers; also mucor is found occasionally in otomycosis.

XV. Dermatomycosis (Dermatophytes).

A. *Obtaining Material for Examination.*

1. Obtain hair and scales from lesions on the scalp with forceps.
2. Clean skin lesions with 70% alcohol and either scrape the edges of the lesion with a scalpel or with the edge of a glass slide.
3. Remove the top of a vesicular lesion with small curved manicure scissors.
4. Obtain friable or discolored areas from infected nails.

B. *Direct Examination.*

1. Place a portion of the material on a slide, add a drop of 10 to 40% KOH, cover with a cover glass, and heat preparation gently over a low flame to clear.
2. *Trichophyton* attacks the hair, skin, and nails.
    a. Infected hairs show chains of small (microides type) or large (megaspore type) arthrospores arranged as follows:
        1) Parallel rows inside hair (endothrix type).
        2) Parallel rows outside of hair (ectothrix type).
    b. In skin and nails Trichophyton appears as segmented, branching mycelial elements, which may or may not break up into arthrospores (same as Microsporum and Epidermophyton).
3. *Microsporum* attacks only the hair and skin.
    a. Infected hairs show a mosaic sheath of small spores surrounding the hair shaft.
    b. In the skin it appears as segmented, branching mycelial elements (same as Trichophyton and Epidermophyton).

4. *Epidermophyton* attacks only the skin and nails and appears as segmented, branching mycelial elements identical with Trichophyton and Microsporum.

C. Cultures.

1. Implant 3 to 4 small fragments of the material short distances apart on each of 3 Sabouraud's glucose agar slants.
2. Incubate at room temperature for at least 2 weeks.
3. Examine daily for growth from the edges of the planted material.
4. As soon as growth appears remove a portion of the aerial growth with a straight sterile transfer wire and place on a slide in a drop of lactophenol cotton blue solution.

Phenol crystals	20 gm.
Lactic acid	20 cc.
Glycerol	40 cc.
Distilled water	20 cc.

Dissolve by heating gently under a hot water tap and then add 0.05 gm. cotton blue.

5. Separate the mycelial mass with dissecting needles and cover with a cover glass.
6. Heat over a low flame to drive out air bubbles and to produce a greater penetration of the stain.

D. *Identification of Cultures.*

1. *Trichophyton.*
    - a. *Gypseum Group* (T. mentagrophytes).
        - 1) Growth is powdery to granular, light buff to rose-tan in color, and may vary from a fluffy, cottony type to velvety and pure white.
        - 2) The under surface of the colony is wine-colored to brownish.
        - 3) Powdery and granular cultures develop numerous microconidia in clusters and singly on the hyphae; also there are usually spirals, nodular bodies, and chlamydospores; see Fig. 24, F.
        - 4) These structures develop less frequently on cottony colonies.
    - b. *Rubrum Group* (T. rubrum).
        - 1) Growth is cottony to velvety but sometimes powdery.
        - 2) The under surface of the colony is reddish to purple; occasionally the aerial mycelia become pinkish in old cultures.
        - 3) Primary cultures develop numerous microconidia in clusters and singly along the hyphae, few macroconidia, chlamydospores, racquet-shaped hyphae, and nodular bodies; see Fig. 25, A.
    - c. *Crateriform Group.*
        - 1) Microscopically the various species show numerous microconidia along the sides of the hyphae, sessile or on sterigmata, and microconidia in grape-like clusters. Chlamydospores and club-shaped terminal swelling of the hyphae are found.
        - 2) *T. tonsurans* colonies are first velvety and white and then powdery cream to yellow color, showing a central depression with an elevated rim forming a crater.
        - 3) *T. epilans* colonies are first somewhat crateriform, but become crumpled with irregular folds, assuming a cerebriform surface. The color is first white but turns cream to yellowish.
        - 4) *T. sabouraudi* colonies are small, hemispheric, and velvety with a few central projections of tufts of hyphae; later they become powdery and heaped. The first white color changes to cream or brownish with a faint tinge of violet.
        - 5) *T. sulfureum* colonies are first velvety and a delicate primrose color with a central red nodule; later they become powdery, folded with a small central crater, and sulfur yellow in color; see Fig. 25, B.
    - d. *Faviform Group.*
        - 1) Microscopically only hyphal swelling numerous chlamydospores, and "favic chandeliers" are seen.
        - 2) *T. schoenleini* colonies are heaped glabrous and cerebriform, yellowish white to brown in color.
        - 3) *T. concentricum* colonies are heaped deeply folded, glabrous, at first white but become deeply brown in the center with a cream-colored powdery periphery.
        - 4) *T. ferrugineum* colonies are heaped deeply folded, glabrous, waxy, and deep reddish yellow to orange.
        - 5) *T. violaceum* colonies are first heaped folded, glabrous, waxy, violet, and later become velvety as aerial mycelia develop.
    - e. *Rosaceum Group* (T. megnini).
        - 1) Colonies are first pure white, cottony to velvety, later becoming pale rose to delicate pink; the under surface of the colony is a "currant violet" or "raspberry rose."
        - 2) Microscopically many microconidia occur in clusters and singly; there are a few macroconidia, racquet hyphae and chlamydospores.

licrosporum.

M. audouini.

1) Colonies are slow-growing, consisting of a closely matted, velvety aerial mycelial growth, light gray to brown in the center with radiating furrows. The under surface of the colony is reddish brown to orange in color.
2) Microscopically a few large, multiseptate, spindle-shaped macroconidia (fuseaux) are seen. Clavate, single-celled microconidia are borne laterally along the hyphae and are sessile or on short sterigmata. Racquet mycelia, pectinate hyphae, nodular bodies, and chlamydospores are present.

). *M. canis.*

1) Colonies develop quickly with a cottony to wooly aerial mycelial growth which becomes powdery and buff to light brown in the center. The under surface of the colony is reddish brown to orange in color.
2) Microscopically numerous large, multiseptate, spindle-shaped, rough, thick-walled macroconidia (fuseaux) are seen as well as racquet hyphae, pectinate hyphae, nodular bodies, and chlamydospores; see Fig. 25, C.

:. *M. gypseum.*

1) Colonies are fast growing, becoming powdery and buff to light brown in color. The under surface of the colony is reddish brown to orange in color.
2) Microscopically numerous large, 4 to 6 septate, ellipsoid, rough-walled macroconidia (fuseaux) are seen as well as racquet mycelia, pectinate hyphae, nodular bodies, and chlamydospores.

Epidermophyton (E. floccosum).

ı. Colonies are first white and granular with a small central tuft of mycelia; later the growth becomes velvety to powdery, with numerous radiating furrows and greenish yellow in color; see Fig. 25, D. Aerial mycelia develop in 3 weeks.

). Microscopically there are large, clavate, multiseptate, smooth, thin-walled macroconidia (fuseaux) singly or in clusters along the hyphae. Chlamydospores are abundant in old cultures.

Interpretation.

Dermatophytes only invade the superficial ayers of the skin, i.e., the epidermis, and do 1ot produce systemic infections.

Tinea pedis is caused by E. floccosum, vari-ous species of Trichophyton, and rarely Mi-rosporum.

3. *Tinea unguium* is caused by E. floccosum, various species of Trichophyton, and C. albicans.
4. *Tinea cruris* is caused by E. floccosum and species of Trichophyton.
5. *Tinea corporis* is caused by various species of Trichophyton and Microsporum.
6. *Tinea imbricata* is caused by T. concentricum.
7. *Tinea barbae* is caused by various species of Trichophyton and Microsporum.
8. *Tinea capitis* is caused by species of Trichophyton and Microsporum.
9. *Tinea favosa* is caused by T. schoenleini, T. violaceum, or M. gypseum.

XVI. Chromoblastomycosis (Verrucous Dermatitis).

A. *Direct Examination.*

1. Place crusts of exudates from the verrucous lesions on a slide, add a drop of 10% KOH, and cover with a cover glass.
2. Heat gently to hasten clearing.
3. The organism appears as a single or clustered, round, thick-walled, dark-brown body; multiplication is by splitting and not by budding; see Fig. 25, E.

B. *Cultures.*

1. Inoculate a Sabouraud's glucose agar slant and keep at room temperature for at least 3 weeks.
2. Hormodendrum pedrosoi and Phialophora verrucosa produce slow growing colonies which are dark brown to black in color; see Fig. 25, F.
3. Hormodendrum compactum produces a slow growing, heaped, brittle colony which is olive black in color.

C. *Identification.*

1. Make a moist preparation of the culture.
2. *Phialophora verrucosa.*
   a. Thin-walled, oval conidia are seen at the cup-shaped tips of the conidiophores.
   b. The conidiophores are single or in groups, terminal or lateral on the hyphae.
3. *Hormodendrum pedrosoi.*
   a. Some conidiophores vary in length and bear conidia in branching chain formation (Hormodendrum-type).
   b. Some conidiophores (knotted clubs) develop as terminal cells or as single lateral branches from the hyphae from which conidia are formed on short protuberances along the length of the conidiophores (Acrotheca-type).
4. *Hormodendrum compactum.*
   a. The conidiophores are terminal and lateral, bearing compact masses of long,

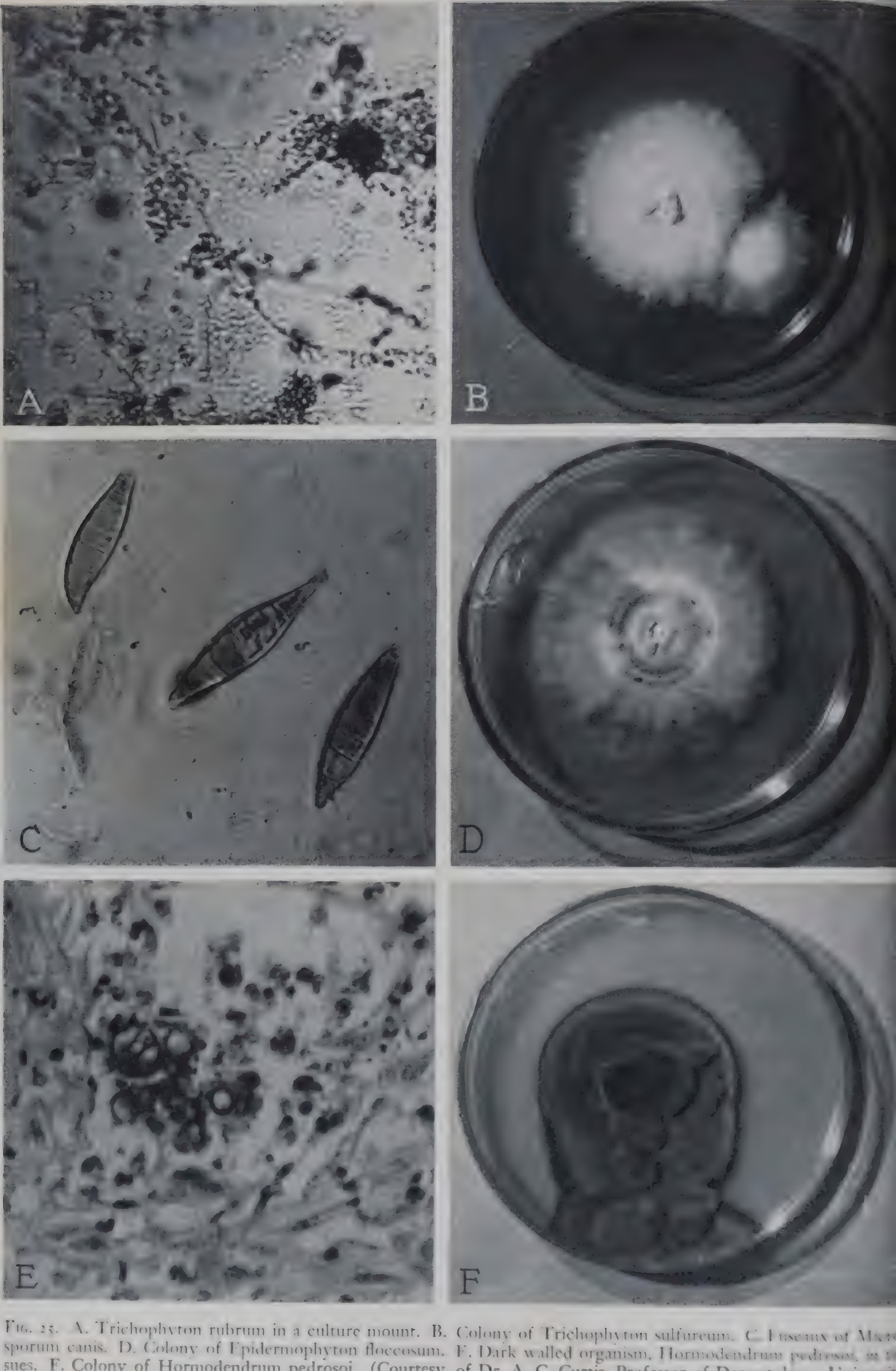


Fig. 25. A. Trichophyton rubrum in a culture mount. B. Colony of Trichophyton sulfureum. C. Fuseaux of Mic
sporum canis. D. Colony of Epidermophyton floccosum. E. Dark walled organism, Hormodendrum pedrosoi, in ti
sues. F. Colony of Hormodendrum pedrosoi. (Courtesy of Dr. A. C. Curtis, Professor of Dermatology, Universit
of Michigan, Ann Arbor.)

branching chains of subspherical conidia. The conidial chains are not as easily broken up as those of H. pedrosoi.

terpretation.

romoblastomycosis or verrucous derma- is is caused by H. pedrosoi, H. compactum, P. verrucosa.

he disease is characterized by warty cutane- s nodules which develop very slowly, form- g prominent papillomatous vegetations hich may or may not ulcerate.

. Tinea Versicolor (Malassezia furfur).

irect Examination.

btain scales from the fawn-colored macules f the skin and place in a drop of 10% KOH n a slide.

over with a cover glass and heat gently over low flame for clearing.

I. furfur appears as clusters of thick-walled, und, and budding forms (3 to 8 microns in iameter) surrounded by short, straight, and ngular fragments of mycelia.

ultures are not necessary.

nterpretation.

I. furfur is the cause of Tinea versicolor.

Tinea versicolor is a chronic asymptomatic uperficial disease characterized by fawn to rownish-colored desquamating macules involving principally the trunk but occasionally the axillae, groin, arms, thighs, neck, face, and scalp.

XVIII. Piedra.

Direct Examination.

Place an infected hair on a slide, add a drop of 10% KOH, and cover with a cover glass.

Piedraia hortai.

- The nodules on the hair are discrete consisting of tightly packed stroma of dark-brown, dichotomously branched hyphae, 4 to 8 microns in diameter.
- The hyphae have numerous septations at such close intervals that they resemble arthrospores.
- A crushed nodule reveals numerous asci containing 2 to 8 single-celled, fusiform, slightly curved ascospores with a single polar filament at one end.

richosporon beigelii.

- The nodules on the hair are variable in size, soft not discrete, and easily detached from the hair.
- The transparent greenish-tinged mycelial mass is seen to extend along the hair as a sheath.
- c. The hyphae tend to be perpendicular to the surface of the hair and segment into round, oval, or rectangular cells, 2 to 8 microns in diameter.
- d. Blastospores are seen in the mycelial mass.

B. ***Cultures.***

1. Make cultures on Sabouraud's glucose agar and incubate at room temperature.
2. *P. hortai.*
   - a. Colonies are greenish black to black, elevated in the center, or are flat, glabrous, or smooth to cerebriform and are adherent to the medium.
   - b. Microscopically there are dark, thick-walled, closely septate hyphae containing numerous chlamydospores or swollen, irregularly-shaped cells.
3. *T. beigelii.*
   - a. Colonies appear rapidly and at first are cream-colored and slimy.
   - b. Later they become finely wrinkled, the center is heaped up, the color is darker, and they are more adherent to the agar.

C. ***Interpretation.***

1. Piedra is a fungus infection of the hair characterized by stony hard nodules along the hair shafts.
2. Piedraia hortai is the cause of black piedra.
3. Trichosporon beigelii is the cause of white piedra.

XIX. Trichomycosis Axillaris.

A. ***Direct Examination.***

1. Place infected hairs on a slide, add a drop of 10% KOH, and cover with a cover glass.
2. Nocardia tenuis produces nodules on the hair which are composed of delicate, short, branching, mycelial elements, 1 micron or less in diameter, which appear to be embedded in mucilaginous material.
3. The red and black varieties show numerous clumps of cocci mixed with the bacillary forms of the fungus.

B. ***Cultures*** are not necessary for diagnosis.

C. ***Interpretation.***

1. Nocardia tenuis is the cause of trichomycosis axillaris.
2. It is an infection of axillary and pubic hairs characterized by the development of yellow, red, or black concretions surrounding the hair shaft.

XX. Erythrasma.

A. ***Direct Examination.***

1. Scrape bits of skin from the infected areas by means of a scalpel or the edge of a glass slide and place on a slide.

2. Add a drop of ether and allow it to evaporate.
3. Add a drop of methylene blue solution or lactophenol cotton blue solution and cover with a cover glass; examine with the oil immersion objective of the microscope.
4. Nocardia minutissima appears as short, delicate, branching filaments, 1 micron or less in diameter, which are easily broken up into smaller bacillary or coccoid forms.

B. ***Cultures*** are not necessary for diagnos

C. ***Interpretation.***

1. Nocardia minutissima is the cause of erythrasma.
2. Erythrasma is a chronic fungus infection the stratum corneum and is characterized superficial lesions in the axillae and gen crural regions, occasionally involving o intertriginous areas.

Serology

General Considerations

ne Wassermann (complement-fixation) test
lly becomes positive two weeks after the
arance of the initial lesion of syphilis, while
flocculation tests are positive earlier. The
ulation tests are also more sensitive and give
her percentage of positive reactions in treated
latent cases of syphilis than does the Wasser-
n test. However, a small percentage of cases
a negative flocculation test and a positive
sermann reaction; this is especially true in
enital syphilis.

diagnosis of syphilis should not be made on
asis of a single doubtful or positive reaction
out the support of a positive history or
cal. evidence. When there is no evidence or
ry of syphilis, the test should be repeated at
4 week intervals for 3 to 6 months in order
le out false reactions. A small percentage of
al individuals have a reagin-like substance
h produces falsely doubtful and positive re-
ns. The following diseases may also give a
reaction, yaws, pinta, bejel, leprosy, malaria,
inia, vaccinoid, measles, mumps, chickenpox,
tious mononucleosis, virus pneumonia, in-
ous hepatitis, scarlet fever, acute tuberculosis,
idosis, lymphogranuloma venereum, lupus
hematosus, relapsing fever, rat bite fever,
l's disease, typhus fever, filariasis and kala-
. The complement-fixation test is less apt to
false positive reactions than the flocculation
in these diseases.

he Wassermann test is a complicated procedure
can be carried out in a satisfactory manner
by one especially trained in the technique.
necessary to carefully titrate the antigen,
oceptor, and complement in order that they
ccurately balanced in the test. The floccula-
tests are less complicated, but the technique
t be carried out with exacting precision.

Sources of Error in Serology Tests

I. Collection of the Blood.

Hemolysis or contamination of the blood by
acteria or chemicals.
Use of oxalate or citrate.
Improper storage of specimens.
Delay in sending specimens to the laboratory.
Mislabeling specimens.
Postmortem specimens.

II. Laboratory Procedure.

1. Insufficient skill and experience of the technician and failure to follow technique exactly as described by the author.
2. Mix-up of specimens.
3. Improperly cleaned glassware.
4. Faulty reagents.
5. Inexact measurements.
6. Inadequate controls.
7. Incorrect reading, recording, or reporting results.

Theory of the Wassermann Test

Three substances are concerned in bacteriolysis and hemolysis. Each one must be present to complete the system.

Bacteriolytic System (First System)

Antigen (invading bacterium) + *Bacteriolytic amboceptor* (in serum of infected person)
+ *Complement* (in serum of any normal animal) = Bacteriolysis.

Hemolytic System (Second System)

Antigen (red blood corpuscles) + *Hemolytic amboceptor* (in serum of animal injected with homologous red blood corpuscles)
+ *Complement* (same as in bacteriolytic system) = Hemolysis.

The important fact of the above formulas is that, while the antigen and amboceptor differ in the two systems, the complement is the same. Whatever the source of the complement, it will serve either for bacteriolysis or for hemolysis. The Wassermann reaction is not a true antigen-antibody combination because the antigen is non-specific, that is, not made from the Treponema pallidum. The antibody (amboceptor) in syphilitic serum is called "reagin" and binds complement to the lipoidal substance in the antigen. As the theory is the same as in the bacteriolytic system we will call the first system in the Wassermann test by that name.

In the application of these principles, it is possible so to adjust the test that any two members of a system being known, the third may be determined qualitatively and (roughly) quantitatively. In the clinical use of the test, one seeks the syphilitic antibody (reagin) present in the patient's serum. To accomplish this, one mixes in a test tube certain amounts of antigen, blood serum (amboceptor), and complement. One of two things will occur:

(1) If the patient suffers from the disease in question and his serum contains the corresponding amboceptor, the complement will be fixed or bound to the antigen by this specific amboceptor and will not be left in a free state.

(2) If the patient's serum does not contain the specific antibody to serve as a connecting link, the complement will remain unbound or free in the fluid.

In either case there will be no visible change to show what has taken place; therefore, it is necessary to add an indicator which will show whether the complement remains free. This is found in the two specific elements of the hemolytic system—red blood corpuscles and hemolytic amboceptor. If free complement is present in the "bacteriolytic system," the hemolytic system is complete and the corpuscles will be hemolyzed. If, upon the other hand, all available complement had been bound to the antigen by the antibody in the "bacteriolytic system," the hemolytic system is not complete and hemolysis can not occur. This shows that the patient's serum contained specific amboceptor and that he probably has the disease in question.

Eagle's Modification of the Wassermann Test

Reagents

I. Sodium Chloride Solution—0.85%.

A. ***Preparation.***

1. Dissolve 8.5 gm. of dried chemically pure sodium chloride in 1000 cc. of distilled water using a volumetric flask.
2. Test by suspending one drop of sheep corpuscles in about 5 cc. of the NaCl solution.
    a. Let stand overnight at room temperature.
    b. The cells should settle to the bottom of the tube leaving a clear, colorless supernatant fluid.

B. ***Used as Follows:***

1. Serves as a diluent throughout the test.
2. The graduates and flasks for the sheep corpuscle suspension, complement, antigen, and amboceptor are rinsed 2 or 3 times with this NaCl solution before being used.

II. Sheep Corpuscle Suspension.

A. ***Collection of Sheep Blood.***

1. Sheep are bled aseptically from the jugular vein.
2. The blood is placed in a sterile flask or b
containing an anticoagulant or beads fo
fibrinating the blood.
3. Boerner and Lukens recommend the fol
ing anticoagulant.

Sodium citrate ....
Glucose ....
1:1000 aqueous solution of merthiolate ......to

    a. Use in proportion of 1 part to 9 par
    blood.
    b. This anticoagulant does not interfere i
    the test because it is removed in the re
    peated washing of the cells.

B. ***Saline Suspension—3%.***

1. Filter about 7 cc. of the sheep blood throug
4 layers of coarse gauze (to remove fibrin i
defibrinated blood is used) into a 50 cc
centrifuge tube and fill with 0.85% NaC
solution.
2. Mix well and centrifuge for 15 to 20 minute
at moderate speed.
3. Decant the NaCl solution and repeat washin
twice. If the supernatant fluid is not clea
washing must be repeated until it is clea
4. The last washing should be made in a 15 cc
centrifuge tube graduated in tenths of a cc
5. Centrifuge for 15 minutes at moderate spee
6. Suspend the cells with 0.85% NaCl solutio
according to the reading on the centrifug
tube to make a 3% suspension which is use
in the test.
7. Always shake before using in the test to se
cure an even suspension of the corpuscle

III. Complement.

A. ***A suitable complement*** must possess tw
properties:

1. Complementary activity or the power of ef
fecting hemolysis through a hemolytic ambo
ceptor.
2. Fixability or the power of being fixed b
antigen and antibody.

B. ***Guinea Pig Serum.***

1. Blood is drawn from the heart of a guine
pig (animal may be anesthetized). It is pos
sible to draw 5 or 6 cc. from each pig with
out harm, if the amount is replaced by th
same volume of sterile 0.85% NaCl solutio
injected into the peritoneal cavity.
2. Complement varies in potency in each pig
so the serum to be used should be a mixtu
obtained from 5 or 6 pigs.
3. Let the blood stand overnight in the refriger
ator.
4. Centrifuge and pour off the clear serum int
a graduate previously rinsed with 0.85% NaC
solution. Measure quantity and add 80 mg

pure sodium chloride per cc. of serum.

he salted serum should be put in a tightly-oppered brown glass bottle and kept in the frigerator.

his salted serum keeps for at least one week. should not be kept more than 7 to 10 days ecause of deterioration.

or use in the test, dilute 1 volume of serum ith 9 volumes of cold distilled water. This equivalent to a 1-10 dilution of unsalted rum with 0.85% NaCl solution.

eep the diluted serum in the refrigerator hen not in use.

/. Hemolytic Amboceptor (Antisheep Hemolysin).

reparation of Amboceptor.

laterials Needed.

Healthy young mature male rabbits. It is better to inject several rabbits because of individual variation in ability to produce antibodies.

. Ten cc. of sterile sheep serum for each rabbit.

. Ten cc. of a sterile 10% suspension of washed sheep corpuscles in 0.85% NaCl solution for each rabbit.

. It is best to use blood of several sheep due to individual variation in antigenic properties.

ensitize each rabbit by giving 5 subcutaneous njections of sheep serum at 48 hour intervals ccording to Schedule A. Thirty minutes efore the third, fourth, and fifth injection, dminister 0.2 cc. of serum (desensitizing dose o prevent shock from the larger amount).

Schedule A

y	Amount of sheep serum injected subcutaneously.
	0.5 cc.
	1.0 cc.
	0.2 cc. followed in 30 min. with 1.3 cc.
	0.2 cc. followed in 30 min. with 1.8 cc.
	0.2 cc. followed in 30 min. with 2.3 cc.

The rabbit is then allowed to rest for 4 days after which it is given 4 intravenous injections of a 10% suspension of washed sheep corpuscles at 48 hour intervals as in Schedule B. Thirty minutes before the second, third, and fourth injections, administer 0.2 cc. of the sheep corpuscle suspension (desensitizing dose to prevent shock from the larger amount).

Five days after the last injection, obtain 1 cc. of blood from the ear vein of each rabbit.

Separate the serum from the blood by centrifugation and titrate each serum according to the directions under titration.

Schedule B

Day	Amount of a 10% suspension of sheep corpuscles injected intravenously.
14	1 cc.
16	0.2 cc. followed in 30 min. with 1.3 cc.
18	0.2 cc. followed in 30 min. with 2.3 cc.
20	0.2 cc. followed in 30 min. with 2.8 cc.

6. If the serum of 1 or more rabbits has a titer of 1-3000 or more, bleed the rabbit from the heart under aseptic conditions on the seventh day after the last injection.
7. Place the blood in the refrigerator overnight and then centrifuge to obtain the serum.
8. The serum may be kept in either of 2 ways.
   a. *Preserved in glycerin.*
      1) Inactivate at 56°C. for 15 minutes on 2 successive days.
      2) Add an equal quantity of chemically pure neutral glycerin and store in small bottles in the refrigerator.
      3) This is a 50% solution of amboceptor so the titer will be only one half of the original titer.
   b. *Sealed in ampules.*
      1) Place in sterile 1 cc. ampules and seal.
      2) Inactivate at 56°C. for 15 minutes on 2 successive days and then store in the refrigerator.
      3) When kept sterile, the amboceptor will keep for several years undergoing a gradual decrease in its titer over this period.

B. *Titration of Amboceptor.*

1. Set up 7 test tubes and add the amboceptor, NaCl solution, sheep corpuscles, and complement according to Table 47.
2. Mix contents by placing thumb on top of tube and inverting; wipe thumb on towel before turning the next tube.
3. Incubate in a 37°C. water bath for 30 minutes.
4. Example of reading titration.
   a. Suppose that the last tube showing complete hemolysis is a 1-3000 dilution.
   b. A unit of amboceptor is 0.4 cc. of a dilution between the 1-3000 and 1-4000 dilutions or approximately 1-3500 dilution.
   c. In the test proper 2½ units are used which from this titration would be 0.4 cc. of a 1-1400 dilution.
5. The amboceptor must be titrated for each set of Wassermann tests to compensate for variation in the fragility of the sheep corpuscles and variation in the strength of the complement.

Table 47. Amboceptor Titration

Tube	1	2	3	4	5	6	7
Amboceptor (1-1000), cc.	0.4	0.27	0.2	0.13	0.1	0.067	[illegible]
NaCl solution (0.85%), cc.	0.8	0.93	1.0	1.07	1.1	1.14	[illegible]
Sheep corpuscle suspension (3%), cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Complement (1-10), cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Dilution of amboceptor*	1-1000	1-1500	1-2000	1-3000	1-4000	1-6000	[illegible]
Example of reading hemolysis	Complete	Complete	Complete	Complete	Partial	Partial	None

*Dilution of amboceptor is that of the ambocepter itself and not that of the final dilution in the titration.

V. Antigen.

A. All containers used for antigen must be absolutely dry.

B. *Preparation of Powdered Beef Heart.*

1. Remove the fat, pericardium, and blood vessels from 6 or more fresh calves' hearts.
2. Grind the muscle in a meat grinder.
3. Add 150 cc. of pure acetone to each 100 gm. of tissue.
4. Keep at room temperature for one hour with frequent shakings.
5. Remove acetone by filtration and add a second portion of 150 cc. of acetone.
6. Shake a few minutes and filter. The acetone filtrates are discarded.
7. Spread tissue out in a large flat pan in a thin layer and dry rapidly with an electric fan.
8. Pulverize the dried tissue in a meat grinder.

C. *Preparation of Tissue Extract.*

1. To each 50 gm. of dried powdered beef heart, add 250 cc. of pure anesthesia ether (5 cc. per gram of powder) and place in the 37°C. incubator for 15 minutes.
2. The ether is filtered off and the extraction repeated with fresh ether for a total of 4 extractions.
3. After the fourth filtration the beef heart powder is washed on the filter paper with 100 cc. of fresh ether. All the ether extracts are discarded.
4. Dry the powder thoroughly on the filter paper.
5. Weigh the powder, place in a flask or bottle, and add absolute alcohol in the proportion of 5 cc. per gram of powder.
6. Shake and let stand at room temperature for 5 days.
7. Filter off the alcohol extract and wash the moist powder on the filter paper with small portions of fresh absolute alcohol until the volume of extract and washings is equal to the original amount of alcohol used for extraction.
8. This clear straw-colored extract is the ba antigen and contains 1.2 to 1.5 per cent soli
9. Keep in a brown glass bottle at room tempe ature.

D. *Cholesterolized Antigen.*

1. Add 6 mg. of cholesterol (Pfanstiehl, c. to each cc. of alcoholic extract.
2. Place in a 37°C. water bath or incubator dissolve.
3. This makes a 0.6% cholesterolized solution.

E. *Titration of Antigen.*

1. *Reagents Needed.*
    a. Cholesterolized antigen.
    b. Sodium chloride solution—0.85%.
    c. Known positive serum—this should co sist of at least 4 different strongly po tive serums mixed together.
    d. Complement—a 1-10 dilution of salt guinea pig serum prepared with cold d tilled water.
    e. Amboceptor—dilution determined by tration as in test proper.
    f. Sheep corpuscle suspension—3%.
2. *Optimum Titration.*
    a. Set up 42 small test tubes in 6 rows 7 tubes each.
    b. Follow Table 48 for contents of t tubes.
3. *Hemolytic Titration.*
    a. Set up 7 small test tubes in a rack at t same time when doing the optimum tit tion.
    b. Follow Table 49 for contents of the tub
4. *Anticomplementary Titration.*
    a. Set up 7 small test tubes in a rack at t same time when doing the optimum a hemolytic titrations of antigen.
    b. Follow Table 50 for contents of the tub
5. Shake all tubes in all three titrations (tubes) and place in the refrigerator for hours.

TABLE 48. OPTIMUM TITRATION OF ANTIGEN

Tube	Antigen dilutions (1) 0.4 cc.	0.2 cc. positive serum dilutions (2)							Complement (1-10), cc.
		Tube 1	2	3	4	5	6	7	
thru 7	1-40	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4
thru 14	1-80	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4
thru 21	1-100	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4
thru 28	1-120	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4
thru 36	1-160	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4
thru 42	1-200	*	1-2	1-4	1-8	1-16	1-32	1-64	0.4

uted strongly positive serum (0.2 cc.).

repare antigen dilutions as follows:

ilution of antigen, cc. . antigen in 19.5 cc. NaCl sol.)	8.0	4.0	3.2	2.7	2.0	1.6
solution (0.85%), cc.	0	4.0	4.8	5.3	6.0	6.4
dilution of antigen	1-40	1-80	1-100	1-120	1-160	1-200

repare positive serum dilutions as follows:

ve serum, cc.	4.0	2.0	1.0	0.5	0.25	0.125	0.062
solution (0.85%), cc.	0	2.0	3.0	3.5	3.75	3.95	4.0
dilution of serum	0	1-2	1-4	1-8	1-16	1-32	1-64

TABLE 49. HEMOLYTIC TITRATION OF ANTIGEN

	Antigen dilutions**						
	1	1-2	1-3	1-4	1-6	1-8	1-12
e antigen, cc.	0.4	0.2	0.13	0.1	0.07	0.05	0.035
solution (0.85%), cc.	0.8	1.0	1.07	1.1	1.13	1.15	1.17
p corpuscle suspension, (1.5% by volume)	0.8	0.8	0.8	0.8	0.8	0.8	0.8
ple of reading hemolysis	Complete	0	0		0	0	0

5% unsensitized sheep corpuscle suspension may be added immediately and the tubes placed in a 37°C. water bath minutes or a sensitized sheep cell suspension may be added at the same time it is added to the optimum and anti-lementary titrations.

ution of antigen is that of the antigen itself and not that of the final dilution in the titration.

TABLE 50. ANTICOMPLEMENTARY TITRATION OF ANTIGEN

	Antigen dilutions						
	1	1-2	1-3	1-4	1-6	1-8	1-12
e antigen, cc.	0.4	0.2	0.13	0.1	0.07	0.05	0.035
solution (0.85%), cc.	0.4	0.6	0.67	0.7	0.73	0.75	0.77
plement (1-10), cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
nple of reading hemolysis	Complete	0	0	Partial	Complete	Complete	Complete

Prepare a sensitized sheep corpuscle suspension 30 minutes before removing tubes from the refrigerator by mixing equal volumes of a 3% sheep corpuscle suspension and amboceptor diluted according to the titration of that day (see test proper). (Thirty cc. of each is enough for all 3 antigen titrations.)

a. Pour the diluted amboceptor into the sheep corpuscle suspension rapidly while shaking the suspension, otherwise a portion of the cells may combine irreversibly with the bulk of the amboceptor, leaving little free for the remainder.
b. Place in the refrigerator for 1 hour.

7. After 4 hours remove the tubes of the optimum, hemolytic, and anticomplementary titrations from the refrigerator and place in a 37°C. water bath for 30 minutes.
8. Remove from water bath and add 0.8 cc. of the well-mixed, sensitized, sheep corpuscle suspension to each tube.
9. Mix contents by placing thumb on top of tube and inverting; wipe thumb on towel before turning the next tube.
10. Again place in a 37°C. water bath for 30 minutes.
11. Remove the tubes from the water bath and read.

F. *Reading of Antigen Titrations.*

1. *Optimum Titration.*
   a. Follow Table 51 for example of titration results.

TABLE 51. EXAMPLE OF OPTIMUM TITRATION OF ANTIGEN

Antigen dilutions	Positive serum dilutions						
	1	1-2	1-4	1-8	1-16	1-32	1-64
1-40	+	+	+	±	0	0	0
1-80	+	+	+	+	±	0	0
1-100	+	+	+	+	+	0	0
1-120	+	+	+	+	+	±	0
1-160	+	+	+	+	+	±	0
1-200	+	+	+	+	+	0	0

+ = no hemolysis.
± = partial hemolysis.
0 = complete hemolysis.

   b. The antigen does not deviate significantly from a 1-120 to a 1-160 dilution. A 1-140 dilution may be used in the test.
2. *Hemolytic Titration.*
   a. Refer to Table 49 for example of titration results.
   b. Hemolysis is complete in the undiluted antigen; therefore, antigen is not significantly more hemolytic than pure alcohol and may be used in dilutions of 1-2 or over without hemolysis by the antigen itself.
3. *Anticomplementary Titration.*
   a. Refer to Table 50 for example of titration results.
   b. Hemolysis is complete in the undiluted antigen, but this hemolysis does not require the presence of either amboceptor or complement. It is due to a hemolytic substance in the antigen. There is no hemolysis in dilutions 1-2 and 1-3 and only partial hemolysis in dilution 1-4.
   c. Having thus been determined, the anticomplementary titer of antigen (1-4 in example) should never be used in more than one fourth of this concentration (1-16 in example given).
   d. The optimum dilution of a good antigen is generally one tenth to one twentieth of the anticomplementary titer.

VI. Fluids to be Tested.

A. *Patient's Serum.*

1. Draw 8 to 10 cc. of blood from the patient's vein.
   a. Blood obtained during an acute febrile illness other than syphilis may give a false positive reaction.
   b. Blood should be obtained before breakfast or 3 or 4 hours after eating. A small number of people will give a false positive reaction unless their blood is obtained when in a fasting state.
2. Put the blood in a sterile test tube and place in the refrigerator until firmly coagulated.
3. Loosen clot and centrifuge 15 minutes at moderate speed.
4. Pour off the serum into a sterile test tube, being careful that the serum is free from corpuscles.
5. Serum must be free from the products of hemolysis of the erythrocytes.
6. Inactivation—human serum contains a variable amount of complement which must be destroyed by heating in a water bath at 56°C. for 15 minutes before using in the test.

B. *Spinal Fluid.*

1. Spinal fluid does not have an appreciable amount of complement; therefore, it is not inactivated.
2. At least 3.6 cc. of fluid is needed for a complete test.
3. Spinal fluid containing more than a trace of blood, a large number of pus cells, or bacterial growth is not satisfactory for the test.

Test Proper

I. Reagents Needed.

- **A. Sodium Chloride Solution**—0.85%.
- **B. Sheep Corpuscles**—3% suspension.
- **C. Complement**—1-10 dilution of guinea pig serum.
- **D. Amboceptor**—1-1000 dilution for the titration.
- **E. Cholesterolized Antigen**—diluted according to previous titration.
- **F. Patient's Serum**—inactivated.
- **G. Spinal Fluid**—not inactivated.
- **H. Known Positive Serum**—inactivated.
- **I. Known Negative Serum**—inactivated.

II. Routine Qualitative Serum Test.

A. *Rack I* (Test Proper).

1. Set up 3 tubes for each patient's serum and follow Table 52 for contents of tubes.

BLE 52. PATIENT'S SERUM—TEST PROPER

Tubes	1	2	3
n dilution, cc.	0.4	0.4	0
t's serum, cc.	0.1	0.2	0.2
ement (1-10), cc.	0.4	0.4	0.4
sol. (0.85%), cc.	0	0	0.4

t up 3 tubes as above, using known positive rum.

t up 3 tubes as above, using known negative rum.

the same rack set up 5 tubes for a comement and antigen control for the test roper.

Follow Table 53 for contents of tubes.

Tube 1 is the antigen control as well as the 1st tube of the complement control.

E 53. COMPLEMENT AND ANTIGEN CONTROL

Tube	1	2	3	4	5
en dilution, cc.	0.4	0.4	0.4	0.4	0.4
lement (1-10), cc.	0.4	0.2	0.13	0.1	0
sol. (0.85%), cc.	0.4	0.6	0.7	0.7	0.8

lace rack with both sets of tubes in the rerigerator for 3 to 4 hours.

ack II (Check on amboceptor titration—his is a duplicate of the complement and ntigen control).

et up 5 tubes following Table 53 for contents f tubes.

lace in refrigerator at same time as Rack I r as soon afterwards as possible.

ack III (Amboceptor Titration).

A titration must be made to determine the ninimal hemolytic quantity (unit) of ambocptor needed for the cell suspension and the complement used that particular day.

Set up 7 tubes and follow Table 47 for the contents of the tubes.

Mix contents by placing thumb on top of tube and inverting; wipe thumb on towel before turning the next tube.

Incubate in water bath at 37°C. for 30 minutes.

Read, following example in Table 47, and prepare a dilution of amboceptor (enough for Racks I and II) containing 2½ units. In the example cited in Table 47, one unit is 1-3500; therefore, 2½ units would be a 1-1400 dilution.

Check on Amboceptor Titration.

As soon as the amboceptor titration is read and the correct dilution of amboceptor (2½ units) is made, mix 5 cc. of this dilution with 5 cc. of a 3% sheep corpuscle suspension. This is a 1.5% suspension of sensitized sheep corpuscles.

2. Place in the refrigerator for 1 hour.
3. At the end of the hour, remove this sensitized corpuscle suspension and Rack II (check on amboceptor titration) and add 0.8 cc. of the well-mixed, sensitized corpuscle suspension to each tube in the rack.
4. Mix by inverting and incubate for 30 minutes in a 37°C. water bath.
5. Remove and read; if the amboceptor titration was correct, the 0.4 cc. of a 1-10 dilution of complement used in the test represents 2 to 2½ times the amount necessary to cause complete hemolysis in 30 minutes.
6. In Rack II the contents of tubes 1 and 2 should be completely hemolyzed, of tube 3 should be partially hemolyzed, tube 4 should show only a very slight amount of hemolysis, and tube 5 no hemolysis.
   a. If only tube 1 shows complete hemolysis, the corpuscles are inadequately sensitized and a lower dilution of amboceptor should be made.
   b. If tubes 1, 2, and 3 show complete hemolysis, an excess of amboceptor has been used and a second lot of amboceptor must be made in the next greater dilution.

E. *Sensitized Sheep Corpuscle Suspension for Test Proper.*

1. Make up the sensitized sheep corpuscle suspension for the test proper (Rack I) using the corrected dilution of amboceptor.
2. Place in the refrigerator for 1 hour.

F. *Test Proper* (Rack I).

1. After the above sensitized sheep corpuscle suspension has been in the refrigerator 30 minutes, remove Rack I which has been in the refrigerator at least 3 hours but not over 4 hours.
2. Place Rack I in the 37°C. water bath for 30 minutes.
3. Remove the sensitized corpuscles 1 hour after placing them in the refrigerator and also at the same time remove Rack I from the water bath.
4. Add 0.8 cc. of the well-mixed, sensitized corpuscle suspension to each tube and mix by inverting.
5. Incubate in a 37°C. water bath for 20 to 40 minutes.
   a. The leeway of 20 minutes is allowed in order to compensate for any slight error in the sensitization of the sheep corpuscles.
   b. Look at the complement control (Table 53) at 20, 30, and 40 minutes.

1) If the contents of tube 2, which contains half the amount of the complement used in the test, is completely hemolyzed at 20 minutes, the rack is removed and the tests read.
2) If hemolysis does not occur in tube 2 until 30 to 40 minutes, the rack is removed when hemolysis does occur. However, if hemolysis does not occur in 40 minutes, the rack is removed and the reason for the incomplete hemolysis is sought.

III. Reading of Results.

A. ***Antigen Control*** **(Tube I of Table 53).**

1. This tube should show complete hemolysis.
2. Properly diluted, the antigen is not demonstrably anticomplementary under the conditions of the test. Failure to hemolyze indicates that the complement is defective and is deteriorating under the conditions of the test.

B. ***Complement Control (Table 53).***

1. The set of tubes incubated along with the serum tests (Rack I) should show the same degree of hemolysis as the tubes in Rack II (check on amboceptor titration) if the same amboceptor dilution was used in both sets of tubes.
2. If hemolysis of the contents of tubes 1 and 2 is incomplete, the complement is defective and deteriorating under the conditions of the test.

C. ***Reading Tests with Patient's Serum.***

1. *Method of Reporting Hemolysis.*

Complete inhibition of hemolysis	++++
75% inhibition of hemolysis	+++
50% inhibition of hemolysis	++
25% inhibition of hemolysis	+
Complete hemolysis	negative

2. The third tube (control which contains no antigen) of each serum test must show complete hemolysis, otherwise the serum is anticomplementary and the test must be repeated.
   a. An anticomplementary serum is one which has the ability to "fix" complement.
   b. If a test with anticomplementary serum is repeated with 0.05, 0.1 and 0.15 cc., it will often yield a clearly negative or positive result.
   c. Each dilution must have a control (without antigen) for anticomplementary substances.

IV. Spinal Fluid.

A. ***Spinal fluid*** is not inactivated.

B. ***Test Proper.***

1. Set up 4 tubes in duplicate in Rack I (Test Proper) for the 4 different amounts of spinal fluid used and the control for each.
2. Follow Table 54 for contents of the tubes.

TABLE 54. SPINAL FLUID—TEST PROPER

Control	Antigen dilution, cc.	0	0	0	0
	Spinal fluid, cc.	1.0	0.5	0.2	0.1
	NaCl sol. (0.85%), cc.	0.2	0.2	0.2	0.2
	Complement (1-10), cc.	0.2	0.2	0.2	0.2
Test	Antigen dilution, cc.	0.2	0.2	0.2	0.2
	Spinal fluid, cc.	1.0	0.5	0.2	0.1
	NaCl sol. (0.85%), cc.	0	0	0	0
	Complement (1-10), cc.	0.2	0.2	0.2	0.2

3. Quantities of reagents are halved in order to conserve spinal fluid. Maximum amount of spinal fluid relative to other reagents is ten times that used in serum tests.
4. Carry through procedure as for serum in the qualitative serum test, using 0.8 cc. of a sensitized sheep corpuscle suspension.

V. Quantitative Serum Test.

A. ***Procedure.***

1. Set up 7 tubes and follow Table 55 for contents.
2. Carry through procedure as for qualitative serum test.

B. ***Reading of Results.***

1. The serum control (1-2 dilution) should show complete hemolysis.
2. Report highest dilution in which there is an inhibition of hemolysis.

VI. Eagle's Complement-fixation Test for Gonorrhea.

A. ***The reagents*** are the same as for the Eagle modification of the Wassermann test except the antigen.

TABLE 55. QUANTITATIVE SERUM TEST (Eagle)

	Serum control	Quantitative test					
Serum dilutions	1-2	1-2	1-8	1-20	1-40	1-80	1-160
Whole serum, cc.	0.2	0.2	0.05				
Serum (1-20), cc. (0.1 cc. + 1.9 cc. NaCl sol.)				0.4	0.2	0.1	0.05
NaCl sol. (0.85%), cc.	0.2	0.2	0.35	0	0.2	0.3	0.35
Antigen dilution, cc.	0	0.4	0.40	0.4	0.4	0.4	0.40
Complement (1-10), cc.	0.4	0.4	0.40	0.4	0.4	0.4	0.40

ntigen.

he antigen is prepared from cultures of a reat many strains of gonococcus and is best urchased from a biological supply company. t is necessary to titrate the antigen at frequent ntervals.

. Make a 1-10 dilution of the antigen in 0.85% NaCl solution and use this dilution in following Table 48 instead of a 1-40 dilution.

b. Also follow Tables 49 and 50 for the hemolytic and anticomplementary titrations.

The amount of antigen used in the test is 1/3 o 1/4 the smallest amount that is anticomplementary, provided that this gives a strongly positive reaction with a known positive serum.

Test Proper.

Inactivate the serum for 30 minutes in a 56°C. water bath.

Set up 4 tubes in duplicate for the 4 different amounts of serum used and the control of each.

Follow Table 56 for contents of the tubes.

TABLE 56. COMPLEMENT-FIXATION TEST FOR GONORRHEA

trol	Antigen dilution, cc.	0	0	0	0
	Serum, cc.	0.2	0.15	0.1	0.05
	NaCl sol. (0.85%), cc.	0.4	0.4	0.4	0.4
	Complement (1-10), cc.	0.4	0.4	0.4	0.4
t	Antigen dilution, cc.	0.4	0.4	0.4	0.4
	Serum, cc.	0.2	0.15	0.1	0.05
	Complement (1-10), cc.	0.4	0.4	0.4	0.4

A known positive and negative serum must be included in the test as well as complement and antigen controls.

Incubate the tubes in the refrigerator for 3 to 4 hours and then incubate for 1 hour in the water bath at 37°C.

Add 0.8 cc. of a sensitized sheep corpuscle suspension to each tube and incubate in the 37°C. water bath as in the Wassermann test.

Results.

Read results in the same manner as in the Wassermann test.

A negative reaction is obtained during the acute stage of gonorrhea.

A positive reaction is obtained in about 35% of the cases of chronic gonorrheal arthritis. One must be cautious in interpreting positive reactions when the serum gives a positive Wassermann reaction.

Kolmer's Complement-fixation Test

Reagents

. **Serum to be Tested**—See Eagle's Modification, page 226.

II. **Sodium Chloride Solution (0.85%)**—See Eagle's Modification, page 222.

III. **Sheep Corpuscle Suspension (2%)**—Prepare as described under Eagle's Modification, page 222.

IV. Antigen.

A. ***All containers*** used for antigens must be absolutely dry.

B. ***Preparation.***

1. Remove the fat, pericardium, and blood vessels from 6 or more fresh calves' hearts.
2. Grind the muscle in a meat grinder.
3. Spread tissue in a large flat pan in a thin layer and dry rapidly with an electric fan.
4. Pulverize the dried tissue in a meat grinder.
5. Place 60 gm. of the powdered beef heart in a flask and add 200 cc. of chemically pure acetone.
6. Stopper tightly and keep at room temperature for 5 days with brief shaking each day.
7. Filter through fat-free filter paper and discard the filtrate.
8. Dry the residue and extract with 200 cc. of chemically pure absolute ethyl alcohol in a tightly stoppered flask or bottle for 5 days at room temperature, shaking daily.
9. Filter through fat-free filter paper with slight squeezing of the tissue, measure the filtrate, and place in a bottle or flask which can be stoppered tightly.
10. Add cholesterol in the proportion of 2 mg. per cc. of filtrate.
11. Dissolve the cholesterol and 2 to 4 gm. of acetone-insoluble lipoids in 40 cc. of ether.
    a. The acetone-insoluble lipoids are obtained by concentrating the first 4 ether extracts used in the preparation of the Kahn antigen (p. 235).
    b. Concentrate to about a fifth of the volume in an evaporating dish and add 3 to 6 volumes of acetone.
    c. After mixing and standing overnight, the supernatant acetone is removed and the residue of acetone-insoluble lipoids is kept in the refrigerator.
12. Add the dissolved cholesterol and acetone-insoluble lipoids to the alcoholic filtrate.

TABLE 57. ANTIGEN TITRATION

Tube	0.5 cc. of serum dilutions	0.5 cc. of antigen dilutions						Complem (2 ful units) in
		Tube 1	2	3	4	5	6	
1 thru 6	1-5 (0.1 cc. serum)	1-80	1-160	1-320	1-640	1-1280	1-2560	1
7 thru 12	1-10 (0.05 cc. serum)	1-80	1-160	1-320	1-640	1-1280	1-2560	1
13 thru 18	1-20 (0.025 cc. serum)	1-80	1-160	1-320	1-640	1-1280	1-2560	1
19 thru 24	1-40 (0.0125 cc. serum)	1-80	1-160	1-320	1-640	1-1280	1-2560	1
25 thru 30	1-100 (0.005 cc. serum)	1-80	1-160	1-320	1-640	1-1280	1-2560	1

13. Shake thoroughly and place in a water bath at 55°C. for 1 hour to aid in the solution of the lipoids.
14. Allow to stand at room temperature for 2 or 3 days, with brief shaking each day, and then filter through fat-free filter paper.
15. Keep at room temperature and do not disturb any sediment.

C. *Titration of Antigen.*

1. It is only necessary to titrate for antigenic activity; the hemolytic and anticomplementary titrations can be omitted if the antigen is made as described above.
2. *Reagents Needed.*
   a. Cholesterolized antigen.
   b. Sodium chloride solution—0.85%.
   c. Known positive serum—this should consist of at least 4 different strongly positive serums mixed together.
   d. Complement diluted so that 1 cc. will equal 2 full units.
   e. Amboceptor diluted so that 0.5 cc. will equal 2 units.
   f. Sheep corpuscle suspension—2%.
3. Make a 1-80 dilution of antigen by adding 0.1 cc. of antigen drop by drop, with shaking between drops, to 7.9 cc. of 0.85% NaCl solution in a large test tube or small flask.
4. Prepare higher dilutions of antigen as follows:

4 cc. of 1-80 + 4 cc. saline = 1-160.
4 cc. of 1-160 + 4 cc. saline = 1-320.
4 cc. of 1-320 + 4 cc. saline = 1-640.
4 cc. of 1-640 + 4 cc. saline = 1-1280.
4 cc. of 1-1280 + 4 cc. of saline = 1-2560.

5. Inactivate 3 cc. of strongly positive syphilitic serum in a water bath at 56°C. for 15 minutes and prepare the following 5 dilutions.

Tube	0.85% NaCl solution in cc.	Serum in cc.	Serum dilution	Serum in 0.5 cc. of dilution
1	4.0	1.0	1-5	0.1 cc.
2	4.5	0.5	1-10	0.05 cc.
3	9.5	0.5	1-20	0.025 cc.
4	2.0	2.0 of 1-20	1-40	0.0125 cc.
5	4.0	1.0 of 1-20	1-100	0.005 cc.

6. Set up 5 rows of small test tubes with 6 i each row and follow Table 57 for contents o tubes.
7. Add 2 control tubes.
   a. Serum control—containing 0.5 cc. of 1- dilution of serum made above and 1 cc of complement containing 2 full units
   b. Hemolytic system control—containing cc. of NaCl solution and 1 cc. of com plement containing 2 full units.
8. Shake the tubes gently and place in the re frigerator at 6 to 8°C. for 15 to 18 hours followed by incubation in a water bath a 37°C. for 10 minutes.
9. Add 0.5 cc. of amboceptor (2 units) and 0. cc. of 2% sheep corpuscle suspension to al tubes.
10. Place thumb on top of each tube and invert wipe thumb on towel before turning th next tube.
11. Place in a water bath at 37°C. for 1 hou and then make readings and chart the result according to Table 58.
12. The 2 control tubes should show complet hemolysis.
13. The optimum amount of antigen to employ in the tests is the amount giving a ++++ reaction with the smallest amount of serum
   a. If two dilutions of antigen give ++++ reactions with the smallest amount o serum as in the 1-320 and 1-640 dilution in Table 58, the optimum amount would be the average of the two or 0.5 cc. of 1-500 dilution.
   b. Before a new antigen is used, the opti mum amount should be checked agains an antigen of proven sensitivity in series of comparative tests employing weakly to moderately positive serums.

V. Hemolytic Amboceptor (Antisheep Hemolysin).

A. ***Preparation*** — See Eagle's Modification page 223.

B. ***Titration.***

1. It is advisable (but not absolutely necessary) to make this titration each time the tests are

TABLE 58. READING OF ANTIGEN TITRATION (Example)

Serum in 0.5 cc.	Antigen in 0.5 cc.					
	1-80	1-160	1-320	1-640	1-1280	1-2560
5 cc.	−	−	++	−	−	−
25 cc.	−	+	++++	++++	++	+
5 cc.	+	++++	++++	++++	++++	+
cc.	+++	++++	++++	++++	++++	++
cc.	++++	++++	++++	++++	++++	+++

conducted.

Reagents Needed.

a. Amboceptor to which glycerin has been added to make a 50% solution and therefore 2 cc. must be added instead of 1 cc. to make a 1-100 dilution.
b. Complement (1-30 dilution).
c. Sheep corpuscle suspension—2%.
d. Sodium chloride solution—0.85%.

Prepare a stock 1-100 dilution of amboceptor by mixing 2 cc. with 94 cc. of NaCl solution and 4 cc. of 5% phenol in NaCl solution. This will keep in the refrigerator for several weeks.

In a series of 10 test tubes, prepare higher dilutions (with thorough mixing) as in Table 59.

TABLE 59. DILUTIONS FOR AMBOCEPTOR TITRATION

Tube	Amboceptor dilution in cc.	0.85% NaCl solution in cc.	Final dilution
1	0.5(1-100)	4.5	1-1,000*
2	0.5(1-1000)	0.5	1-2,000
3	0.5(1-1000)	1.0	1-3,000*
4	0.5(1-1000)	1.5	1-4,000*
5	0.5(1-1000)	2.0	1-5,000*
6	0.7(1-3000)	0.7	1-6,000*
7	0.7(1-4000)	0.7	1-8,000*
8	0.5(1-5000)	0.5	1-10,000
9	0.5(1-6000)	0.5	1-12,000
10	0.5(1-8000)	0.5	1-16,000

This dilution is used for further dilutions.

5. Set up a series of 10 tubes in a rack and follow Table 60 for contents of tubes using amboceptor dilutions made in Table 59.
6. Mix the contents of each tube and incubate in the water bath at 37°C. for 1 hour.
7. Read the highest dilution of amboceptor that gives complete hemolysis as 1 unit.
8. Dilute the amboceptor so that 0.5 cc. equals 2 units; for example, if the unit equals 0.5 cc. of 1-6000, two units equal 0.5 cc. of 1-3000.
9. Dilute only enough of the 1-100 dilution of amboceptor for the tests for 1 day (see Table 61) and keep in the refrigerator when not in use.
10. One unit of amboceptor should equal 0.5 cc. of a 1-4000 dilution or higher.

TABLE 60. AMBOCEPTOR TITRATION

Tube	0.5 cc. of amboceptor dilutions	Complement (1-30) in cc.	0.85% NaCl solution in cc.	2% sheep corpuscle suspension in cc.
1	1-1,000	0.3	1.7	0.5
2	1-2,000	0.3	1.7	0.5
3	1-3,000	0.3	1.7	0.5
4	1-4,000	0.3	1.7	0.5
5	1-5,000	0.3	1.7	0.5
6	1-6,000	0.3	1.7	0.5
7	1-8,000	0.3	1.7	0.5
8	1-10,000	0.3	1.7	0.5
9	1-12,000	0.3	1.7	0.5
10	1-16,000	0.3	1.7	0.5

TABLE 61. PREPARATION OF AMBOCEPTOR FOR TEST PROPER

1 unit in 0.5 cc. of	2 units would be 0.5 cc. of	Dilute 1 cc. of the 1-100 dilution with the following amounts of 0.85% NaCl solution.
1-1,000	1-500	4 cc.
1-2,000	1-1000	9 cc.
1-3,000	1-1500	14 cc.
1-4,000	1-2000	19 cc.
1-5,000	1-2500	24 cc.
1-6,000	1-3000	29 cc.
1-8,000	1-4000	39 cc.
1-10,000	1-5000	49 cc.

VI. Complement.

A. *Preparation.*

1. Obtain guinea pig serum as described under Eagle's Modification, page 222.
2. The guinea pig serum may be preserved by adding 240 mg. of sodium chloride for each cc. of serum and kept in the refrigerator.
3. If salted complement is used, the 1-30 dilution used in the titration is made by adding cold distilled water; if a greater dilution is necessary for the test proper, first dilute to 1-30 with cold distilled water and then add cold 0.85% NaCl solution to make the dilution desired.

TABLE 62. COMPLEMENT TITRATION

Tube	Complement (1-30) in cc.	Antigen dilution in cc.	0.85% NaCl solution in cc.		Amboceptor (2 units) in cc.	2% sheep corpuscle suspension in cc.	
1	0.20	0.5	1.3	Shake tubes gently and place in water bath (37°C.) for 1 hr.	0.5	0.5	Shake tubes gently and place in water bath (37°C.) for 1 hr.
2	0.25	0.5	1.3		0.5	0.5	
3	0.30	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2		0.5	0.5	
5	0.40	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1		0.5	0.5	
7	0.50	0.5	1.0		0.5	0.5	
8	0.55	0.5	1.0		0.5	0.5	
9	0.60	0.5	0.9		0.5	0.5	
10	none	none	2.5		none	0.5	

B. *Titration.*

1. *Reagents Needed.*
   a. Complement—a 1-30 dilution made by adding 0.3 cc. of guinea pig serum to 8.7 cc. of cold 0.85% NaCl solution.
   b. Antigen diluted according to the antigen titration.
   c. Sodium chloride solution—0.85%.
   d. Amboceptor diluted so that 0.5 cc. contains 2 units.
   e. Sheep corpuscle suspension—2%.
2. Set up 10 small test tubes in a rack and follow Table 62 for contents of tubes.
3. After the tubes have been incubated the second hour, determine the *exact unit* by reading the smallest amount of complement just giving complete hemolysis. This should not be more than 0.5 cc. or less than 0.3 cc. If less than 0.3 cc., it is necessary to use 0.3 cc. as the exact unit as less complement falls below the absolute minimum and is likely to be unsatisfactory.
4. A *full unit* is the next higher tube which contains 0.05 cc. more complement.
5. Two full units are used in the test proper. Example:

   Exact unit = 0.3 cc.
   Full unit = 0.35 cc.
   2 full units = 0.7 cc.
6. The complement is diluted for the test proper so that 1 cc. contains 2 full units.
   a. To calculate the dilution, divide 30 by the amount equal to 2 full units.
   b. Example:

   $\frac{30}{0.7} = 43$ or 1 cc. of 1-43 dilution of complement.

   c. For additional examples see Table 63.
7. The dilution is made with cold 0.85% NaCl solution unless salted guinea pig serum is used and then it must be diluted as follows:
   a. If dilution is 1-30 or less, dilute with cold distilled water.
   b. If dilution is greater than 1-30, make a 1-30 dilution with cold distilled water and further dilute with cold 0.85% NaCl solution.
8. Keep in the refrigerator when not in use.

TABLE 63. DILUTION OF COMPLEMENT FOR TEST PROPER

Exact unit in cc.	Full unit in cc.	Two full units in cc.	Dilution to use
0.30	0.35	0.7	1-43
0.35	0.40	0.8	1-37
0.40	0.45	0.9	1-33
0.45	0.50	1.0	1-30
0.50	0.55	1.1	1-27
0.55	0.60	1.2	1-25
0.60	0.65	1.3	1-23

Test Proper

I. Qualitative Method.

A. *Serum.*

1. Set up 3 test tubes for each patient's serum (inactivated) and add the following, mixing the NaCl solution and serum of the first tube well before transferring to the second and third tubes.

	0.85% NaCl solution in cc.	Patient's serum in cc.	Amount of serum present
Tube 1	0.9	0.6	0.2 cc.
Tube 2	0.5	0.5 from Tube 1*	0.1 cc.
Tube 3	0.5	0.5 from Tube 1	0.2 cc.**

*Discard 0.5 cc. from tube 2.
**Tube 3 is the serum control and contains 0.2 cc. of serum in 1 cc.

2. Set up the following control tubes for each series of tests.
   a. Three test tubes, as above, containing a known positive serum (inactivated).
   b. Three test tubes, as above, containing a known negative serum (inactivated).
   c. Antigen control—see Table 64.
   d. Hemolytic control—see Table 64.
   e. Sheep corpuscle control—see Table 64.

TABLE 64. QUALITATIVE TEST (Kolmer)

ube	Contents of tubes in cc. of saline-serum mixture	Antigen dilution in cc.		Complement (2 full units) in cc.		Amboceptor (two units) in cc.	2% sheep corpuscle suspension in cc.	
1	0.5 (0.2 cc. serum)	0.5	Let stand 10-30 min. at room temperature.	1.0	Shake tubes gently and place in the refrigerator at 6 to 8°C. for 15-18 hrs. Then incubate in water bath at 37°C. for 10-15 min.	0.5	0.5	Shake tubes well and incubate in the water bath at 37°C. for 1 hr.*
2	0.5 (0.1 cc. serum)	0.5		1.0		0.5	0.5	
3	1.0 (0.2 cc. serum)	none		1.0		0.5	0.5	
gen rol	0.5**	0.5		1.0		0.5	0.5	
olytic rol	1.0**	none		1.0		0.5	0.5	
uscle rol	2.5**	none		none		none	0.5	

re sensitive readings are obtained 10 minutes after the antigen, hemolytic, and serum controls show complete olysis.

. of 0.85% NaCl solution only.

Follow Table 64 for the addition of antigen, complement, amboceptor, and sheep corpuscle suspension and time of incubation.

Spinal Fluid.

Set up 3 test tubes and add 0.25 cc. of 0.85% NaCl solution to the second tube and 0.5 cc. to the third.

Add 0.5 cc. of spinal fluid to the first and third tubes and 0.25 cc. to the second tube. The third tube is the control and should not contain antigen.

Follow Table 64 for the addition of antigen, complement, amboceptor, and sheep corpuscle suspension and time of incubation.

If prezone or nonspecific reactions are obtained, repeat the tests and add 0.2 cc. of a 50% solution of egg albumin in sterile NaCl solution to each tube containing spinal fluid.
a. The egg albumin solution is made by breaking a fresh egg and separating the white from the yolk.
b. Pick out the heavy particles from the white or filter through several layers of gauze.
c. Measure, beat briefly, and add an equal volume of sterile 0.85% NaCl solution.
d. This solution will keep in the refrigerator for about 2 weeks.
e. An alternate method is to make a 10% solution of the egg albumin in sterile NaCl solution and use this for diluting the complement so that 1 cc. contains 2 full units.

Interpretation of Results of Qualitative Tests.

The serum, hemolytic, and antigen controls should show complete hemolysis; the sheep corpuscle control should show no hemolysis.

2. Read the test according to inhibition of hemolysis as described on page 228.
3. Report as follows:
   a. Strongly positive: ++++ in first or second tubes. Examples: 4 4; 4 3; 3 4.
   b. Moderately positive: ++++ in first tube only. Examples: 4 2; 4 1.
   c. Weakly positive: +++, ++, or + in one or both tubes. Examples: 3 1; 2 1; 3 —; 2 —; 1 —.
   d. Doubtfully positive: + in either tube.
   e. Negative: complete hemolysis in both tubes.

II. Quantitative Method.

A. *Serum.*

1. Set up 6 test tubes for each patient's serum (inactivated) and add the following, mixing the contents of each tube well before transferring to the next tube.

Tube	0.85% NaCl solution in cc.	Patient's serum in cc.	Amount of serum present
1	0.9	0.6	0.2 cc.
2	0.5	0.5 from Tube 1	0.1 cc.
3	0.5	0.5 from Tube 2	0.05 cc.
4	0.5	0.5 from Tube 3	0.025 cc.
5	2.0	0.5 from Tube 4*	0.005 cc.
6	0.5	0.5 from Tube 1	0.2 cc.**

*Discard 2 cc. from tube 5.
**Tube 6 is the serum control and contains 0.2 cc. of serum in 1 cc.

2. Set up the following control tubes for each series of tests.
   a. Six test tubes, as above, containing a known positive serum (inactivated).
   b. Six test tubes, as above, containing a known

negative serum (inactivated).
   - c. Antigen control—see Table 64.
   - d. Hemolytic control—see Table 64.
   - e. Sheep corpuscle control—see Table 64.
3. Follow Table 64 for the addition of antigen, complement, amboceptor, and sheep corpuscle suspension and for time of incubation.

B. *Spinal Fluid.*

1. Set up 6 test tubes for each spinal fluid and add the following, mixing the contents of each tube well before transferring to the next tube.

Tube	0.85% NaCl solution in cc.	Spinal fluid in cc.	Amount of spinal fluid present
1		0.5	0.5 cc.
2	0.5	0.5	0.25 cc.
3	0.5	0.5 from Tube 2	0.125 cc.
4	0.5	0.5 from Tube 3	0.0625 cc.
5	0.5	0.5 from Tube 4*	0.03125 cc.
6	0.5	0.5	0.5 cc. **

*Discard 0.5 cc. from tube 5.
**Tube 6 is the spinal fluid control and contains 0.5 cc. of spinal fluid in 1 cc.

2. Follow Table 64 for the addition of antigen, complement, amboceptor, and sheep corpuscle suspension and time of incubation.
3. See spinal fluid qualitative test for prezone or nonspecific reactions.

C. *Interpretation of Results of Quantitative Test.*

1. Read in the same manner as the qualitative test.
2. Report as follows:
   - a. Very strong positive: ++++ in third, fourth, or fifth tubes. Examples: 4 4 4 4 4; 4 4 4 4 —; 4 4 4 2 —; 4 4 4 — —; 3 4 4 — —.
   - b. Strongly positive: ++++ in the second tube. Examples: 4 4 3 1 —; 4 4 2 — —; 3 4 2 — —; 4 4 — — —.
   - c. Moderately positive: ++++ in the first tube. Examples: 4 3 1 — —; 4 2 — — —; 4 — — — —.
   - d. Weakly positive: +++ or less in 1 or more tubes. Examples: 3 2 1 — —; 2 1 — — —; 1 — — — —.
   - e. Doubtfully positive: ± in the first tube. Example: ± — — — —.
   - f. Negative: Complete hemolysis in all tubes.

III. Kolmer's Complement-fixation Test for Gonorrhea.

A. ***The reagents*** are the same as for the Kolmer complement-fixation test for syphilis except the antigen.

B. *Antigen.*

1. The antigen is prepared from cultures
great many strains of gonococci and is
purchased from a biological supply co
2. For the anticomplementary titration
12 test tubes in a rack and follow T
for contents of tubes. The hemolytic
trol should show complete hemolysis and
corpuscle control no hemolysis.
3. If a known positive serum is available,
antigenic titration is advisable but not a
lutely essential.
4. The serum must be inactivated for 30
utes at 56°C.
5. For the antigenic titration set up 12 test
in a rack and follow Table 66 for cont
of tubes. The serum and hemolytic con
should show complete hemolysis and
corpuscle control no hemolysis.
6. An antigen may be used in an amount equ
lent to about 1/3 or 1/4 of its anticom
mentary unit.
7. If an antigenic titration is made, the amo
used should be at least 2 to 10 antigenic
providing this is no more than 1/3 of
anticomplementary unit.
8. The antigen should be retitrated every 2
3 months.

C. *Test Proper.*

1. Follow either the qualitative or quantita
method for Kolmer's complement-fixation
for syphilis using gonococcus antigen inst
of beef heart antigen.
2. The readings should be made 10 minutes a
complete hemolysis of the antigen and se
controls.
3. For interpretation of results, see Eagle's c
plement-fixation test for gonorrhea, page

Kahn Flocculation Test

I. Apparatus Needed.

A. *Test Tubes.*

1. Test tubes should have an inside diamete
0.9 cm. and length of 7.5 cm.
2. Vials for the preparation of antigen dilu
should have an inside diameter of 1.5 cm.
length of 5.5 cm.

B. *Pipettes.*

1. As many 1 cc. pipettes graduated in 0.01
as serums to be tested.
2. One 1.5 cc. pipette graduated in 0.05 cc.
antigen.
3. One 0.25 cc. pipette graduated in 0.025
for antigen.

TABLE 65. ANTICOMPLEMENTARY TITRATION OF GONOCOCCUS ANTIGEN

Tube	Antigen dilutions, 0.5 cc.	0.85% NaCl solution in cc.	Complement (2 full units) in cc.		Amboceptor (two units) in cc.	2% sheep corpuscle suspension in cc.	
1	undiluted	0.5	1.0	Shake tubes gently and place in the refrigerator at 6 to 8°C. for 15-18 hrs. Then incubate at 37°C. for 30 min.	0.5	0.5	Shake tubes well and incubate in the water bath at 37°C. for 1 hr.; then read.
2	1-2	0.5	1.0		0.5	0.5	
3	1-3	0.5	1.0		0.5	0.5	
4	1-4	0.5	1.0		0.5	0.5	
5	1-6	0.5	1.0		0.5	0.5	
6	1-8	0.5	1.0		0.5	0.5	
7	1-10	0.5	1.0		0.5	0.5	
8	1-12	0.5	1.0		0.5	0.5	
9	1-16	0.5	1.0		0.5	0.5	
10	1-20	0.5	1.0		0.5	0.5	
nolytic trol	0	1.0	1.0		0.5	0.5	
puscle trol	0	2.5	0		0	0.5	

TABLE 66. ANTIGENIC TITRATION OF GONOCOCCUS ANTIGEN

Tube	Antigen dilutions, 0.5 cc.	Positive serum in cc.	Complement (2 full units) in cc.		Amboceptor (two units) in cc.	2% sheep corpuscle suspension in cc.	
1	1-10	0.5	1.0	Shake tubes gently and place in the refrigerator at 6 to 8°C. for 15-18 hrs. Then incubate in water bath at 37°C. for 30 min.	0.5	0.5	Shake tubes well and incubate in the water bath at 37°C. for 1 hr.; then read.
2	1-20	0.5	1.0		0.5	0.5	
3	1-40	0.5	1.0		0.5	0.5	
4	1-60	0.5	1.0		0.5	0.5	
5	1-80	0.5	1.0		0.5	0.5	
6	1-100	0.5	1.0		0.5	0.5	
7	1-200	0.5	1.0		0.5	0.5	
8	1-300	0.5	1.0		0.5	0.5	
9	1-400	0.5	1.0		0.5	0.5	
um ntrol	0.5 cc. saline	0.5	1.0		0.5	0.5	
molytic ntrol	1.0 cc. saline	0	1.0		0.5	0.5	
rpuscle ntrol	2.5 cc. saline	0	0		0	0.5	

One 0.125 cc. pipette graduated in 0.0125 cc. for antigen.

One 10 cc. pipette for NaCl solution.

Test Tube Racks.

II. Reagents Required.

Sodium Chloride Solution—0.9%.

Serum.

Prepare as for the Wassermann test.

Inactivate by heating for 30 minutes at 56°C. in a water bath.

Antigen Extract.

Remove the fat, pericardium, and blood vessels from 6 or more fresh calves' hearts.

2. Grind the muscle in a meat grinder.
3. Spread tissue in a large flat pan in a thin layer and dry rapidly with an electric fan.
4. Pulverize the dried tissue in a meat grinder.
5. Extract 25 gm. of the powder 4 times with anesthesia ether by shaking the bottle for 10 minute periods with 100, 75, 75, and 75 cc. of ether respectively.
6. After each extraction the ether is filtered off through filter paper, applying gentle pressure to the beef heart by means of a spatula. When no more ether passes through the funnel, return the beef heart to the original bottle.
7. After the last extraction transfer the moist beef heart to a large sheet of filter paper and dry until there is no odor of ether.
8. Weigh the dried beef heart and place in the same bottle which has been dried and freed of ether odor.
9. Add 5 cc. of 95% alcohol for each gram of powder.
10. Shake the bottle for 10 minutes and leave at room temperature for 3 days without shaking.
11. At the end of this period, shake the bottle for 5 minutes and filter extract into a dry bottle.

12. Add 6 mg. of chemically pure cholesterol for each cc. of extract.
13. Dissolve the cholesterol by rotating the bottle in a water bath at 37°C.
14. When all the cholesterol has been dissolved, filter the antigen.
15. Allow to stand at least one day before titration.
16. All stoppers, rubber or cork, that come in contact with the antigen must be covered with high grade tin foil.

III. Titration of Antigen.

A. *Determination of Antigen Dilution.*

1. Add 1 cc. of cholesterolized antigen to each of five vials.
2. To five similar vials add 0.8, 0.9, 1.0, 1.1, and 1.2 cc., respectively, of 0.9% NaCl solution.
3. Empty each NaCl solution vial into a given antigen vial and immediately pour the mixture quickly back and forth at least 6 times to permit thorough mixing. (Do not wait to drain the vial.)
4. Let stand 30 minutes.
5. Test, in NaCl solution, the solubility of the flocculation in each of the 5 antigen dilutions as follows:
    a. Pipette 0.05, 0.025, and 0.0125 cc. amounts, respectively, of each antigen dilution into 3 standard tubes for the test, using a 0.1 cc. pipette graduated in 0.001 cc. Deliver the quantities to the bottom of the tubes.
    b. Add 0.15 cc. of 0.9% NaCl solution to each tube.
    c. Shake all tubes vigorously for 3 minutes.
    d. Add 0.5 cc. of 0.9% NaCl solution to each tube.
    e. Examine each tube to determine whether the original antigen dilution flocculation has gone back into solution.
    f. The antigen-NaCl solution mixture, containing the smallest amount of NaCl solution in proportion to antigen and having a flocculation which dissolves in NaCl solution, represents the end-point of this titration and determines the dilution of antigen for the tests.

B. *Determination of Sensitiveness of Antigen.*

1. Compare the new antigen with a known "standard Kahn antigen."
2. Select 10 inactivated serums as follows:
    a. Two known negative serums.
    b. Two strongly positive serums.
    c. Six weakly positive serums.
3. Run duplicate tests with the new and old antigen and compare the results.
4. If the two antigens give comparable results, the new antigen is considered a "stan Kahn antigen."

IV. Method for Standard Test.

A. *Preparation of Standard Antigen Dilut*

1. Have all apparatus set up before prepa antigen dilution.
2. Antigen dilution may be prepared for tests at a time.
3. If the antigen titer is 1:1, measure 1.2 cc. antigen into one antigen vial and 1.2 cc. 0.9% NaCl solution into another.
4. Pour the NaCl solution into the antigen immediately pour the mixture back and fo at least 6 times. Do not wait to drain vial.
5. Allow this antigen dilution to stand 10 m utes at room temperature before pipetti The antigen is only good for 30 minutes af mixing.

B. *Pipetting of Antigen.*

1. Pipette antigen dilution for 10 tests at a ti immediately before the serum is to be add
2. Shake the antigen well (closing the mouth the vial with the thumb) before pipetting frequently during pipetting.
3. Pipette the following amounts and deliver the bottom of the tubes.
    a. In the first row, 0.05 cc., with a 1.5 pipette.
    b. In the second row, 0.025 cc., with a 0 cc. pipette.
    c. In the third row, 0.0125 cc., with a 0. cc. pipette.

C. *Pipetting Serum.*

1. Add 0.15 cc. of each inactivated serum to e of the 3 antigen dilution quantities by me of a 1 cc. pipette graduated in 0.01 cc.
2. As soon as the serums have been added for tests, shake the rack sufficiently to ins mixing the serum with antigen dilution.

D. *Controls in First Rack.*

1. *Antigen Control.*
    a. Pipette the 3 regular antigen amounts add 0.15 cc. of 0.9% NaCl solution.
    b. All 3 tubes should show no flocculatio
2. *Positive Serum Control.*
    a. Add 0.15 cc. of a known positive ser (inactivated) to the 3 regular anti amounts.
    b. All 3 tubes should show a heavy floc lation.
3. *Negative Serum Control.*
    a. Add 0.15 cc. of a known negative ser (inactivated) to the 3 regular anti amounts.

All 3 tubes should show no flocculation.

Shaking.

After pipetting serums for 20 tests, or one rack, shake vigorously for 3 minutes.

If a shaking machine is used, it should oscillate 275 to 285 times per minute with a stroke of one and one-half inches.

If there are only a few tests, the antigen-serum mixture should stand 3 to 7 minutes before the final 3 minute shaking period.

Addition of Sodium Chloride Solution.

After shaking, add 1 cc. of 0.9% NaCl solution to each tube in the front row containing 0.05 cc. of antigen and 0.5 cc. of 0.9% NaCl solution to each tube in the other two rows.

Also add 0.9% NaCl solution to the antigen, negative, and positive serum controls in the same manner.

Shake sufficiently to mix contents of tubes.

Reading Results.

About 5 minutes after the addition of the NaCl solution, read the tests by holding the tubes in a slanting position 2 to 3 inches in front of the concave surface of a microscope mirror. Daylight or artificial light may be used.

Look at the 3 tubes of each test very carefully for flocculation.

Interpret a definite flocculation suspended in a clear medium as 4 plus and proportionately weaker reactions as 3, 2, and 1 plus respectively.

Read each tube separately and total the pluses of the 3 tubes and report as follows:

a. A total of 6 to 12 pluses in the 3 tubes is reported as positive.
b. A total of 2½ to 5½ pluses inclusive is reported doubtful.
c. A total of 2 pluses or less is reported negative. (A ± reaction is considered as ½ in totaling.)

V. Method for Quantitative Test.

Method.

Prepare 9 serum dilutions according to Table 67.

Prepare the antigen suspension in the same manner as for the qualitative test and let stand 10 minutes.

Pipette 0.01 cc. of the antigen dilution into each of 10 tubes.

Add 0.15 cc. of undiluted serum and a similar amount of each of the 9 serum dilutions to each of the 10 tubes respectively.

Shake for 3 minutes and then add 0.5 cc. of 0.9% NaCl solution to each tube.

6. Shake to mix.
7. Read as described under standard test and determine the number of Kahn units.

B. *Determination of Kahn Units.*

1. A definite flocculation (++++, +++, or ++) is recorded as positive while a weak reaction (+ or ±) is considered negative.
2. If the serum gives a positive reaction only in the undiluted state, it is reported to contain Kahn units as indicated by the plus signs (4 units, 3 units, 2 units, respectively).
3. The potency of any serum which is positive on dilution is determined according to the following formula:

S = 4D.
S = serum potency in terms of Kahn units.
D = the highest dilution ratio giving a positive reaction.

TABLE 67. PREPARATION OF SERUM DILUTIONS

Tube	0.9% NaCl solution in cc.	Serum in cc.	Serum dilution
1	0.5*	0.5*	1-2
2	0.5	0.5 of 1-2	1-4
3	0.5	0.5 of 1-4	1-8
4	0.5	0.5 of 1-8	1-16
5	0.5	0.5 of 1-16	1-32
6	0.5	0.5 of 1-32	1-64
7	0.5	0.5 of 1-64	1-128
8	0.5	0.5 of 1-128	1-256
9	0.5	0.5 of 1-256	1-512

*A smaller amount (0.4 or 0.3 cc.) may be used if not enough serum is available to use 0.5 cc.

4. For example, if serum dilution 1-4 is positive and 1-8 negative, the serum contains 4 × 4 or 16 Kahn units.
5. Serums showing 4 or more units are reported: "Positive, 4 Kahn units," or "positive, 16 Kahn units," etc.

Supplementary Kahn Tests

When serum is so excessively rich in antibody that it requires a relative excess of antigen suspension to give maximum flocculation, a supplementary test is run. This is indicated (1) when flocculation is marked in the first tube and weak or negative in the second and third tubes of the standard test, and (2) when the three tubes in the standard test show a border-line reading such as ±, ±, ±; +, +, +; or perhaps a ++, ++, ++. If the supplementary examination does not show a positive reaction, the three tube standard test which showed flocculation in the first tube must be considered a weak or doubtful reaction.

I. Test Using Excess Antigen.

A. ***Method***—set up 2 tubes and add contents as follows:

	Tube 1	Tube 2
Antigen suspension, cc.	0.02	0.02
Serum, cc.	0.02	0.04
Shake tubes 3 minutes.		
0.9% NaCl solution, cc.	0.3	0.3
Shake to mix.		

B. ***Interpretation.***

1. Tubes 1 and 2, or at least 2, should show definite flocculation reactions if the serum is strongly positive.
2. If this supplementary test is also border-line or questionable, it is probably a nonspecific flocculation due to conditions other than syphilis.

II. Partial Quantitative Test.

A. ***Method.***

1. Dilute serum 1 - 5, 1-10, and 1 - 20 with 0.9% NaCl solution and test each dilution with antigen suspension in a proportion of 15 to 1.
2. Set up 3 tubes and add contents as follows:

	Tube 1	Tube 2	Tube 3
Antigen suspension, cc.	0.01	0.01	0 .
Diluted serum, cc.	0.15 (1-5)	0.15 (1-10)	0.15 (1-20)
Shake tubes 3 minutes.			
0.9% NaCl solution, cc.	0.5	0.5	0.5
Shake to mix.			

B. ***Interpretation.***

1. If 1 or more tubes show definite flocculation, the serum can be considered positive.
2. Some serums are so potent that they may not show a positive reaction until further dilution is made, as a 1-40 or 1-80.

Kahn Verification Test

The verification test consists in performing Kahn tests at 37°, 21°, and 1°C. Since the test is done routinely at approximately 21°C., the basic differential temperatures are 37° and 1°C.

I. Method for 37°C.

A. ***Mixing Serum and Antigen at 37°C.***

1. Place a 10 cc. antigen and as many 1 cc. pipettes as needed in a 37°C. incubator warm.
2. Place a rack containing the following in 37°C. water bath:
   a. Three small test tubes for each serum be tested. (Do no more than ten at one time.)
   b. Three test tubes for a positive serum control.
   c. Three test tubes for a negative serum control.
   d. Three test tubes for an antigen control.
   e. Serum to be tested, previously inactivated.
   f. Known positive serum, previously inactivated.
   g. Known negative serum, previously inactivated.
   h. Sodium chloride solution (0.9%)—enough for all tests.
   i. Kahn antigen first mixed at room temperature as usual and then transferred to a small Kahn tube and stoppered with a cork wrapped in tin foil.
3. After 15 minutes use the warm pipettes to add the following to the tubes in the rack which is kept in the water bath.
   a. The usual amounts of antigen suspension (0.05, 0.025 and 0.0125 cc.) for each test and controls.
   b. The usual amounts of serum (0.15 cc.) to the tubes for each test, including the positive and negative control.
   c. Sodium chloride solution (0.15 cc.) to the antigen control tubes.
4. Shake the rack for 10 seconds and leave in the water bath for 5 to 10 minutes.

B. ***The test is finished at room temperature.***

1. Remove the rack from the water bath (except the 0.9% NaCl solution which is left in the bath) and proceed as in the standard test.
   a. Shake for 3 minutes.
   b. Add warm 0.9% NaCl solution as in the standard test.
   c. Shake to mix.
2. Read immediately and record as for the standard test.

II. Method for 1°C.

A. ***Ice bath*** improvised with chopped ice.

B. ***Procedure***—each step described for 37°C. is carried out at 1°C.

III. Correlation of Readings.

A. An increase in reaction at 37°C. with no change or a decrease at 1°C. is considered a syphilitic type of reaction.

n increase at 1°C. with a decrease or no ange at 37°C. is conisdered a biologic type reaction; this type of reaction is that nor- lly given by the serum of many animals d is nonspecific.

o definite change is reported as inconclusive.

Kline Flocculation Test

I. Apparatus Needed.

ipettes.

s many 1 cc. pipettes graduated into hun- redths as serums to be tested.

capillary pipette which will deliver a drop qual to about 0.008 cc. (62 drops per 0.5 cc.). A syringe and 25 gauge needle may be used it delivers drops of the correct size.)

lass Slides with Paraffin Rings.

se large glass slides, 2 by 3 inches.

lean with Bon Ami paste by letting it dry nd wiping it off with a cloth.

araffin rings (14 mm. in diameter) are made n the slides by dipping a special loop into moking paraffin (about 120°C.), draining uickly at one point, and then pressing it n the slide. If the paraffin is too hot, it will pread too much and the chambers will be oo small.

reparation of Special Loop.

. Wind a soft iron wire (No. 28) twice around a test tube (15 mm. outside diameter) forming a double loop and leaving a double shaft about an inch in length.

. Twist the 2 shafts together to within a quarter of an inch of the free ends.

. Remove the looped wire from the test tube and fasten a No. 12 linen thread to the free ends of the shaft.

. Make several twists around the shaft and then wind the loop tightly with the thread and continue up the shaft to fasten the thread to the 2 ends of the wire by twisting them.

. Bend loop at right angles to the shaft and clamp the shaft with a straight hemostat.

II. Reagents Required.

odium Chloride Solution—0.85%.

repare with distilled water having a pH of bout 6.

Water of the right pH will give a lilac color hen 1 drop of chlorphenol red indicator LaMotte) is added to 0.25 cc. of water.

erum.

repare as for the Wassermann test.

2. Inactivate by heating for 30 minutes at 56°C. in a water bath.

C. *Cholesterol Solution—1%.*

1. Place 1 gm. of cholesterol (Pfanstiehl, c. p.) in a 200 cc. glass-stoppered bottle and add 100 cc. of absolute ethyl alcohol.
2. Place in a 50 to 56°C. oven for 45 minutes with gentle shaking for a few minutes at 15 minute intervals to dissolve the cholesterol.

D. *Antigen Extract.*

1. Place 200 gm. of dried beef heart powder (prepared in same manner as for Kahn antigen) in a 2 liter Erlenmeyer flask.
2. Add 1 liter of absolute ethyl alcohol (99 plus %).
3. Stopper flask with a cork covered with tin foil and shake vigorously by hand at intervals for 2 hours; the total shaking should be 20 to 30 minutes.
4. The extract is filtered into a liter cylinder through good grade filter paper of medium texture.
5. During filtration the mixture is stirred with a wooden tongue depressor and, toward the end, pressed with the cork until the powder is quite dry.
6. The extract (about 775 cc.) is placed in the refrigerator at 8 to 10°C. for 24 hours during which time a fairly heavy white precipitate settles out.
7. The extract is again filtered and the filtrate placed in a large evaporating dish.
8. The filtrate is concentrated to about 35 cc. on a water bath or by an air heater (bathroom heater) at 45 or 50°C.
   a. This temperature is determined by a thermometer bulb within the extract.
   b. During the evaporation of the alcoholic extract, an irregular festoon appears at the periphery.
   c. When the extract reaches the proper concentration, the festoon disappears and the margin of the concentrated extract is sharp. (Place in a 37°C. incubator overnight if necessary.)
9. Pour quickly 500 cc. of c. p. acetone, heated in an incubator to 50°C., into the concentrated extract. It is important that the acetone be at this temperature but not above it.
10. To be sure of complete precipitation, the evaporating dish is placed in the incubator at 37°C. for 15 minutes after which the acetone is carefully decanted, leaving a soft brown wax adhering to the side of the dish.
11. The dish is placed in a water bath or incubator at 45 to 50°C. for about 30 minutes,

or until the odor of acetone is no longer detectable.

12. The wax is then worked together and placed in a glass-stoppered bottle.
13. Add 80 cc. of absolute ethyl alcohol which has been kept in the paraffin oven at 56°C. for 30 minutes.
14. After a few minutes shaking, the bottle is placed in a paraffin oven at 56°C. for 30 minutes to dissolve as much of the wax as possible. Shake gently after it is in the oven 15 minutes.
15. Remove the bottle from the oven and shake for a few minutes and then place it in the refrigerator at 8 to 10°C. for 45 minutes.
16. Filter and evaporate the filtrate at 45 to 50°C. (water bath or air heater) until a soft brown wax (antigen wax) is left.
17. Place the wax in a weighed glass-stoppered bottle and for each gram add 10 cc. of absolute ethyl alcohol.
18. After the bottle is shaken for a few minutes, it is placed in the paraffin oven at 56°C. for 30 minutes and then shaken a few minutes again. The wax is not completely soluble in the alcohol.
19. The slightly turbid solution is placed at 8 to 10°C. for about an hour and is then filtered.
20. The resultant clear filtrate is the antigen and contains about 8.75% of the alcohol-treated acetone-insoluble wax.
21. The antigen is kept at room temperature and should be good for at least 6 months.
22. *Purification.*
    - a. In a warm room (70°F. or more), pipette 5 cc. of distilled water (pH about 6) into a liter Erlenmeyer flask.
    - b. Add 10 cc. of antigen, as prepared above, by holding the tip of the pipette against the neck of the flask.
    - c. Rotate the flask on a flat surface for 1 minute with considerable vigor and then rotate more gently for 1 minute controlling the motion to collect the wax, which has precipitated out, toward the center.
    - d. Allow the flask to stand until the wax settles and sticks to the bottom (about 15 seconds).
    - e. Carefully decant the fluid by slowly tilting the flask forward on a flat surface to a level just permitting the fluid to run out.
    - f. Continue the tilting until the flask is completely inverted.
    - g. Allow the flask to drain on a piece of filter paper for 2 to 3 minutes.
    - h. Dry the inner wall of the flask with a
    gauze sponge for a distance of
    inches and place with the open
    in a 50°C. incubator for 1 hour or
    - i. At the same time place a 30 cc.
    stoppered bottle, a 10 cc. pipette,
    bottle of absolute alcohol in the
    incubator.
    - j. The following extracting process
    ried out inside the incubator.
    - k. After 1 hour or more, pipette 5
    the warm ethyl alcohol into the flask
    taining the wax.
    - l. Fifteen minutes later shake the flask
    the wax is completely dissolved
    alcohol (1 to 5 minutes).
    - m. Tilt the flask to collect the fluid and
    pipette it into the bottle.
    - n. Again add 5 cc. of absolute alcohol
    flask, shake about 15 seconds, and
    the fluid into the bottle.
    - o. Add absolute alcohol to the flask
    third time in such quantity (about
    cc.) that, when it is transferred, the
    in the bottle will be 10 cc. It is
    vantage to have previously marked
    10 cc. level on the bottle with a wax
    cil.
    - p. Stopper the bottle and transfer to a
    incubator until the following
    after which it is kept at room
    ture.
    - q. The purified antigen solution may at
    be somewhat turbid but will clear
    standing several hours. If an
    quantity of wax settles out, the
    should be filtered.
    - r. This procedure may be carried out
    smaller quantities of antigen and
    tionately smaller quantities of
    small Erlenmeyer flasks.

III. Test Proper.

A. *Antigen Emulsion.*

1. Pipette 0.85 cc. of distilled water (pH
6) into a 1 ounce bottle.
2. Allow 1 cc. of a 1% solution of
in absolute ethyl alcohol to run down the
of the bottle. The bottle is held by the
and gently rotated for 20 seconds.
3. Add in the same manner 0.1 cc. of the
stopper, and shake vigorously against the
per for 1 minute.
4. Add rapidly 2.45 cc. of 0.85% NaCl
(pH about 6), stopper, and shake again
vigorously for 1 minute.
5. The emulsion, when examined under the
croscope at a magnification of about 120
shows numerous very fine particles
clumps.

lace the bottle containing the emulsion in water bath at 35°C. for 15 minutes. It is ady for use as soon as heated.

kept at room temperature, it is satisfactory use for 48 hours after preparation.

lethod.

ipette 0.05 cc. of serum into a paraffin ring n a glass slide.

o the serum add 1 drop of the antigen emulion from a capillary pipette or a syringe and eedle which delivers 62 drops per 0.5 cc.

Rotate the slide on a flat surface for 4 minites.

he results are read at once through the nicroscope at a magnification of about 120 imes (low power objective, eyepiece 12) vith the light cut down as for urine sedinents.

he results are reported according to the legree of clumping and the size of the clumps. Compare with a known positive and negative erum set up at the same time.

Any spilling from the chamber makes the eaction unsatisfactory and the test on the erum concerned should be repeated.

Mazzini Flocculation Test

I. Apparatus Needed.

Pipettes.

Six 5 cc. graduated pipettes, one for the test proper and 5 for the antigen titration.

As many 1 cc. pipettes graduated in hundredths to the tip as serums to be tested.

Bottles.

Round bottles of 30 cc. capacity for preparing 3.4 cc. of antigen suspension.

A round bottle of 15 cc. capacity for preparing half-quantity (1.7 cc.) of antigen suspension.

Syringe—a 5 cc. syringe with a 25 gauge needle for delivering antigen.

Glass Slides with Paraffin Rings—prepare as described under Kline Flocculation Test, page 239.

II. Reagents Required.

Serums.

Prepare as for the Wassermann test.

Inactivate in a 56°C. water bath for 30 minutes.

Antigen Extract.

Prepare powdered beef heart as described under Kolmer's antigen, page 229.

Place 20 gm. of powdered beef heart, 10 gm. of powdered egg yolk (may be obtained from Bessire and Co., Inc., Indianapolis, Indiana), and 200 cc. of anesthesia ether in a 500 cc. wide-mouth, glass-stoppered bottle.

3. Shake in a mechanical shaker for 5 minutes or by hand for 15 minutes.
4. Filter through a fat-free 24 cm. filter paper into a liter flask.
5. Repeat this ether extraction 4 additional times using 100 cc. of ether each time.
6. A new filter paper is used for each filtration and all the ether filtrates are collected in the liter flask; the combined filtrates will be used later.
7. After the last extraction spread the moist powder on new filter paper and allow to dry completely.
8. Place the dried powder in a 500 cc. glass-stoppered bottle, add 80 cc. of absolute ethyl alcohol, and agitate in a mechanical shaker for 4 hours or leave at room temperature for 3 days with 5 minute periods of shaking 3 times each day.
9. Filter into a 100 cc. wide-mouth, glass-stoppered bottle and discard the powder.
10. The combined ether extract from the first extractions is treated as follows:
    a. Place in a large evaporating dish (8.5 inches in diameter) and evaporate the ether by placing the dish in a water bath at 55°C. until no ether bubbles rise to the surface of the liquid.
    b. Warm 100 cc. of acetone (Merck's Reagent) to 55°C. in an incubator or water bath and pour rapidly into the concentrated ether extract.
    c. Stir thoroughly with a steel spatula and immediately decant into two 50 cc. centrifuge tubes and centrifuge at 2000 revolutions per minute for 5 minutes.
    d. Pour the acetone off and discard.
    e. Add 10 cc. of fresh acetone to each tube and stir the acetone-insoluble lipoids with a glass rod; place the palm of the hand over the mouth of the tube and invert several times.
    f. Pour off the supernatant acetone and discard.
    g. Collect the acetone-insoluble lipoids with a spatula and add to the alcoholic extract in 9 above.
11. Place the bottle containing the alcoholic extract and acetone-insoluble lipoids in a 55°C. water bath for 30 minutes, shaking gently at frequent intervals; place in the refrigerator for 30 minutes to cool.
12. Filter through a fat-free 12.5 cm. filter paper of fine texture and the antigen filtrate is ready to titrate.

13. Any precipitate that appears on standing is removed by filtration.
14. Keep tightly stoppered in a brown bottle at room temperature.

C. ***Cholesterolized Alcohol—1%.***

1. Place 500 mg. of c. p. cholesterol (Pfanstiehl, ash free) in a 100 cc. glass-stoppered bottle and add 50 cc. of absolute ethyl alcohol.
2. Heat in a 55°C. water bath with agitation at frequent intervals until the cholesterol is completely dissolved.
3. Filter through Whatman No. 50 filter paper.

D. ***Buffered Salt Solution.***

Sodium chloride, c.p.	2.025 gm.
Sodium phosphate, dibasic (anhydrous Na_2HPO_4)	0.169 gm.
(or 0.425 gm. of crystalline $Na_2HPO_4 \cdot 12H_2O$)	
Potassium phosphate, monobasic (KH_2PO_4)	0.050 gm.
Double distilled water	250.0 cc.
N HCl	0.8 cc.
Formalin (Merck's Reagent)	0.25 cc.

1. Filter and check the pH which should be 6.3 to 6.4.
2. Keep in a glass-stoppered pyrex bottle.
3. If the solution should become contaminated with any debris, it should be filtered.

III. Titration of Antigen.

A. ***Preparation of Cholesterolized Antigen.***

1. Set up in a rack of 5 test tubes labeled 1 to 5.
2. Place 0.1 cc. of the antigen extract directly into the bottom of each tube.
3. Add 0.9 cc., 1.4 cc., 1.9 cc., 2.4 cc., and 2.9 cc. of 1% cholesterolized alcohol to each of the 5 tubes respectively and mix the contents thoroughly.
4. Tube 1 contains antigen in a 1-10 ratio, tube 2 a 1-15, tube 3 a 1-20, tube 4 a 1-25, and tube 5 a 1-30.

B. ***Preparation of Antigen Suspensions.***

1. Label five 30 cc. bottles 1-10, 1-15, 1-20, 1-25, and 1-30 respectively.
2. Pipette 3 cc. of buffered salt solution into each of the 5 bottles.
3. With a 1 cc. pipette graduated to the tip, measure 0.4 cc. (reading from the bottom of the pipette) of the 1-10 cholesterolized antigen; hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the buffered saline from the pipette held in the right hand.
4. Draw the suspension into the pipette and blow in and out 2 or 3 times.
5. Add in the same manner the 1-15, 1-20, 1-25, and 1-30 cholesterolized antigen to the bottle with the corresponding label.
6. Cork the bottles and let stand at room t perature for 3 hours.

C. ***Trial of Antigen Suspensions.***

1. At the end of 3 hours, shake the 1-10 susp sion gently from bottom to cork and ba times and then pour into a 5 cc. syringe fi with a 25 gauge needle.
2. Place 0.05 cc. of each of 30 negative seru into corresponding paraffin rings.
3. Discharge 1 drop of the 1-10 suspension i each of the 30 serums.
4. Rotate the slide with a circular, slightly "je ing" motion for 4 minutes at 120 rotatio per minute.
5. Examine the results under the low power o jective of the microscope with subdued lig There should be numerous, uniformly d persed, very small round or slightly elongat particles of lipoid-cholesterol complex wi out the slightest clumping.
6. Repeat using each of the other 4 suspensio with the same serums and record the resul
7. Suspensions showing spontaneous clumpi of the particles can not be used in the t proper.

D. ***Determination of the Antigen Titer.***

1. Select 10 partially positive serums (1, 2, ar 3 plus) and place 0.05 cc. of each into co responding paraffin rings.
2. Discharge 1 drop of the 1-10 antigen su pension into each serum and rotate for 4 mi utes at 120 rotations per minute.
3. Examine as described above and record re sults.
4. Proceed in the same manner with each sus pension of antigen which did not show an clumping with the negative serums.
5. The antigen titer selected for the test prope is the one showing the maximum sensitivity that is, the suspension containing the highes lipoid-cholesterol ratio which gives a positiv reaction but does not cause the least clumpin in the presence of negative serums.
6. Enough cholesterolized antigen of the prope titer can be prepared for 1 month; keep tight ly stoppered.

IV. Test Proper.

A. ***Antigen Suspension.***

1. Prepare an antigen suspension from a choles terized antigen of the ratio determined by the previous titration.
2. Cork the bottle and allow to stand at room temperature for 3 hours (or place the bottle in the refrigerator at 6° to 8°C. for 15 min-

es; however, this is not as satisfactory).
his suspension may be used for 24 hours.

ualitative Test with Serum.

ake the antigen suspension gently from the
ottom to cork and back 10 times and trans-
r to a 5 cc. syringe with a 25 gauge needle.
pette 0.05 cc. of each patient's serum into
corresponding paraffin ring.
pette 0.05 cc. of a known positive and nega-
ve serum in adjacent paraffin rings for con-
ols.
ischarge 1 drop of antigen suspension into
ch serum.
otate the slide for 4 minutes at 120 rota-
ons per minute.
xamine each ring macroscopically to make
rtain that no serum has "jumped" the ring
nd contaminated another.
xamine each serum with a low power ob-
ctive with subdued light, being careful to
xamine the periphery of the rings for clumps,
r occasionally the clumps become compact
nd locate at the periphery.
ecording results:
No clumping=negative.
. Very small clumps=+.
. Small clumps=++.
. Medium size clumps=+++.
. Large clumps=++++.

9. *Zone Reactions.*
   a. *Type I*—insufficient antigen.
      1) Irregular aggregates appear with the smaller clumps predominating.
      2) Add another drop of antigen and rotate slide for another 4 minutes and read.
   b. *Type II*—excess of reagin.
      1) Very few small clumps are present.
      2) Repeat test using serial dilutions of serum as in the quantitative test.

C. ***Quantitative Test with Serums.***

1. Set up 6 test tubes in a rack and number 1 through 6.
2. Add 0.5 cc. of 0.85% NaCl solution to each tube.
3. Add 0.5 cc. of serum to tube 1 and mix; transfer 0.5 cc. to tube 2 and mix; transfer 0.5 cc. to tube 3 and mix; continue through tube 6.
4. Repeat test as described under qualitative test for each dilution.
5. Report results in terms of actual quantity of serum tested, for example:

0.0250 cc. = ++++
0.0125 cc. = ++++
0.0062 cc. = ++++
0.0031 cc. = ++
0.0015 cc. = +
0.0007 cc. = negative.

Blood Groups

General Considerations

There is no laboratory procedure in which the results of erroneous technique or interpretation are more disastrous than in the typing and cross-matching of blood. The direct result of a mistake may be fatal.

Blood Groups

I. The Four Major Blood Groups.

A. All human bloods have been divided into 4 groups with certain subgroups. This grouping is the result of the interaction of agglutinins found in the serum or plasma and agglutinogens present in the erythrocytes. Corpuscles containing agglutinogen A are agglutinated strongly by serums containing agglutinin a and less strongly by serums containing both agglutinins a and b (ab). Corpuscles containing agglutinogen B are agglutinated strongly by serums containing agglutinin b and less strongly by serums containing both agglutinins a and b (ab). Corpuscles containing both agglutinogens A and B (AB) are agglutinated strongly by serums containing both agglutinins a and b (ab) and less strongly by serums containing agglutinin a or b. Corpuscles containing no agglutinogens, group 0, are not agglutinated by any serums. See Table 70.

B. There are 3 different classifications of the 4 blood groups. The Jansky classification is used chiefly in Europe. The Moss classification has been used very extensively in this country. The International or Landsteiner classification uses the letters of the agglutinogens in the corpuscles to designate the group. This classification is now used universally as it is self explanatory and less apt to lead to confusion. See Table 68 for a correlation of the different classifications and group incidence.

II. Subgroups.

A. Groups A and AB can be divided further into subgroups. The corpuscles of group A blood contain either A_1 or A_2 agglutinogens and the blood is designated subgroup A_1 or A_2 according to the agglutinogen present.

The agglutinogens A_1 and A_2 are also
basis of the subgroups of AB which are ca
A_1B and A_2B. Approximately 1 in 5 in
viduals of Group A or Group AB belong
subgroup A_2 or subgroup A_2B. There
two main varieties of "a" agglutinin pres
in the serum of groups B and O: (1) anti
agglutinin proper or "a" reacting with bo
agglutinogens A_1 and A_2 with approximat
equal intensity, (2) anti-A_1 agglutinin or "
which reacts with agglutinogen A_1 but n
with agglutinogen A_2.

TABLE 68. BLOOD GROUPS AND THEIR INCIDEN

Equivalent blood groups			Agglutinogen in erythrocytes	Agglutinin in serum	Per c of indi vidua
Landsteiner	Moss	Jansky			
AB	I	IV	AB	none	6
A	II	II	A	b	40
B	III	III	B	a	12
O	IV	I	none	ab	42

B. The subgroups are important, because a
glutinogens A_1 and A_2 differ considerably
sensitivity; also their reactivity is often wea
er in group AB while the agglutinogen
does not vary in sensitivity. The relati
sensitivity of the A agglutinogens in the
subgroups is as follows: $A_1 > A_1B > A_2 > A$
For this reason, if a weak group B serum
used for typing, it is possible that a patie
of group A_2 or A_2B may be typed wrong
due to the low reactivity of the A_2 aggluti
gen. Group A_2 would be called group
and group A_2B would be called group B.

C. For routine work it is not necessary to dete
the subgroups. Cross-matching of the bloo
of the patient and prospective donor must
done routinely and this avoids the danger
undetected subgroups.

III. Agglutinogens M and N.

A. Humans can be divided into 3 distinct typ
according to whether their erythrocytes co
tain agglutinogen M (50%), N (20%),
both M and N (30%). These agglutinoge

e entirely different from agglutinogens A d B and are not encountered in routine ood typing, because anti-M or anti-N agutinins are extremely rare in human serum. rom hundreds of thousands of typings there ave been only 7 cases of anti-M agglutiins reported, 4 of which were presumably atural and 3 were produced by immunizaon through blood transfusions. No cases aving anti-N agglutinins have been reported.

yping for groups M, N, and MN is not a outine procedure and is usually done only or medicolegal purposes, see Table 73. The nti-M and anti-N typing serums are obtained rom rabbits that have been immunized with roup O erythrocytes of known M and N roups. The production of good typing seums of this kind is a difficult and compliated procedure.

IV. Agglutinogen P.

Humans can be divided into 2 groups accordng to whether their erythrocytes contain the gglutinogen P or whether it is absent. About 75% of the people are P positive and 25% P negative. Cases in which human serum conains anti-P agglutinins are very rare; so agglutinogen P does not interfere with routine blood typing.

Serum containing anti-P agglutinins for typing is obtained from normal animal serum, particularly from pigs and horses which frequently contain these agglutinins.

V. Rh and Hr Factors—See page 249.

Blood Typing

I. General Considerations.

Time Required for Agglutination.

Some bloods show stronger agglutination reactions than others, characterized especially by the speed in agglutinating.

Enough time must be allowed so that less sensitive cells will agglutinate.

Temperature Necessary for Typing.

The tests should be performed at room temperature or at 37°C. to rule out the possibility of cold agglutinins interfering in the test.

Rarely a patient's serum will agglutinate his own corpuscles at room temperature (autoagglutination) but not at body temperature. Autoagglutinins have been found in patients with multiple myeloma, paroxysmal hemoglobinuria, cirrhosis of the liver, hemolytic anemia, and trypanosomiasis.

C. *Controls.*

1. Although not necessary, a group O serum may be used in typing as a control for the group A and group B typing serums.
2. A drop of the blood cell suspension alone on the slide will rule out any clumping of the cells that may be mistaken for agglutination when the suspension is mixed with the typing serums.

II. Solutions.

A. *Sodium Chloride Solution—0.85%.*

B. *Blood Cell Suspension—1 to 2%.*

1. Puncture the ear or finger and allow 1 or 2 drops of blood to fall directly into a small test tube containing about 3 cc. of 0.85% NaCl solution.
2. Mix immediately by inverting the tube a few times.

C. *Known Group A and B Typing Serums.*

1. Collect blood aseptically by venipuncture from healthy individuals of groups A and B between the ages of 20 and 30 who are known to have high titered serum.
2. Place in the refrigerator overnight.
3. Centrifuge immediately after taking out of the refrigerator and separate the serum with aseptic precautions.
4. Inactivate the serum for 15 minutes at 56°C. to destroy the complement.
5. After obtaining the titer of the serum, it may be preserved by one of the following methods:
   a. Keep in 1 cc. sterile glass-stoppered bottles in the refrigerator.
   b. Distribute in capillary tubes after which the ends are sealed in a flame. Store in the refrigerator.
6. *Method for determining titer of serum.*
   a. For each serum (A and B) prepare 5 dilutions as follows, mixing the contents of each tube thoroughly before transferring a portion to the next tube.

Tube	0.85% NaCl solution in cc.	Serum in cc.	Dilution of serum
1	0.8	0.2	1-5
2	0.5	0.5 of 1-5	1-10
3	0.5	0.5 of 1-10	1-20
4	0.5	0.5 of 1-20	1-40
5	0.5	0.5 of 1-40	1-80

   b. Prepare a 2.5% suspension of group A (preferably A_2) blood in 0.85% NaCl solution.
   c. Prepare a 2.5% suspension of group B blood in 0.85% NaCl solution.

d. Set up 10 small test tubes and follow Table 69 for contents.

Table 69. Titration of Typing Serums

Tube	0.2 cc. of serum	0.2 cc. of blood cell suspension	Final dilution
1	1-5 A	B	1-10
2	1-10 A	B	1-20
3	1-20 A	B	1-40
4	1-40 A	B	1-80
5	1-80 A	B	1-160
6	1-5 B	A	1-10
7	1-10 B	A	1-20
8	1-20 B	A	1-40
9	1-40 B	A	1-80
10	1-80 B	A	1-160

e. The tubes are allowed to stand with occasional shaking for 2 hours at room temperature after which they are examined macroscopically for clumping.

f. A satisfactory A serum should have a titer of 1-40 (final dilution) or higher.

g. A satisfactory B serum should have a titer of 1-80 (final dilution) or higher if the subgroup of the A cells used is not known. If the cells used are of subgroup A_2, then a titer of 1-40 is satisfactory.

h. If the typing serums are diluted with an equal amount of 0.85% NaCl solution before using in the slide method for typing, the titers must correspond to the next greater dilution.

III. Slide Method for Typing.

A. *Materials Needed.*

1. Known group A and group B typing serums.
2. A 1 to 2% saline suspension of the blood cells of the patient.
3. Glass slides with 2 paraffin rings similar to those used in the Kline serology test (p. 239).

B. *Method.*

1. Label one end of the slide A and the other end B.
2. With a capillary pipette place a small drop of serum of group A in the ring marked A and with a different capillary pipette add a small drop of unknown corpuscles; mix thoroughly with a pin.
3. In the ring marked B, place a small drop of serum of group B (using a clean pipette) and a small drop of unknown corpuscles and mix thoroughly with a pin.
4. Carefully rotate the slide on a flat surface for 5 minutes to secure thorough mixing.
5. Examine for agglutination of the corpuscles with the low power objective of the microscope. When the reaction is complet agglutinated corpuscles appear as brick granules to the unaided eye. Do not c fuse rouleaux formation or false clum at the edges with agglutination. See Fig.
6. If agglutination has not taken place, e ine every 5 minutes for 30 minutes, 1 for children, mixing each time by tilting slide back and forth to prevent the cor cles from settling in the center.
7. To prevent evaporation, cover the slide the top of a Petri dish when not examin it.
8. If no agglutination has taken place in minutes (or 1 hour for children), the r tion can be considered negative.
9. By comparing the results obtained group A and group B serums with Table or Fig. 26, the group of the unknown c can be determined.
10. See sources of error in blood typing un V.
11. Although blood groups are not fully dev oped at birth, the blood of young infa should be typed, because agglutinogens always present in the cells and agglutin are present in the serum in 50% of the cas
12. To determine whether a person of group belongs to subgroup A_1 or subgroup retype the cell suspension using group serum in which the agglutinin for A_2 a glutinogen has been removed by absorpti with A_2 corpuscles. This serum will on agglutinate cells of group A_1.

Table 70. Reaction Between the Aggluti gens and Agglutinins of the Blood Groups

Cells	Serum			
	AB (−)	A (b)	B (a)	O (ab)
AB	−	+	+	+
A	−	−	+	+
B	−	+	−	+
O	−	−	−	−

+ = agglutination of cells.
− = no agglutination of cells.
Capital letters = blood groups and also agglutinogens in t blood cells.
Small letters = agglutinins in the serum.

IV. Test Tube Method for Typing.

A. *Materials Needed.*

1. Known group A and group B typing serum
2. A 1 to 2% saline suspension of the blood ce of the patient.
3. Sodium chloride solution—0.85%.
4. Small test tubes (7 mm. diameter).

Method.

Label 1 test tube A and another B.

Place 1 drop of 0.85% NaCl solution in each.

Add 1 drop of undiluted group A typing serum to the tube labeled A.

Add 1 drop of undiluted group B serum to the tube labeled B.

Add 1 drop of the patient's blood cell suspension to each tube.

Mix by shaking and then allow the tubes to stand at room temperature for 5 minutes.

Centrifuge the tubes for 1 minute at 2000 revolutions per minute.

Shake gently to suspend the cells or clumps and examine macroscopically for clumping. (The concave mirror of the microscope may be used to magnify small clumps.)

If in doubt about the presence of clumping, place a drop of the suspension on a slide and examine with the low power objective of the microscope.

Compare results with Table 70 or Fig. 26 to determine the blood group.

V. Sources of Error in Blood Typing.

False Negative Reactions.

The use of typing serum of low titer due either to a low original titer or to a gradual decrease resulting from the aging of the serum.

Low sensitivity of agglutinogens of the erythrocytes frequently occurs in children, in stored blood, and with agglutinogen A_2 of subgroups A_2 and A_2B. The most common errors are to call group A_2, group O, and group A_2B, group B. Because of this low agglutinogen reactivity, it is necessary to have typing serums of high titer.

The masking of agglutination by rapid hemolysis when fresh high titer typing serums are used that have not been inactivated to destroy the complement.

The use of a too dilute cell suspension. If the cells are too far apart, the agglutinating force will not bring them together and clumping will not occur.

The use of a too concentrated cell suspension, which may result in the absorption of the agglutinins from the serum before agglutination occurs; that is, there are not enough agglutinins to go around.

The preparation is not allowed to stand a sufficient length of time for agglutination to take place.

The failure to label the typing serums correctly or the interchange of serums in performing the test. This is the most dangerous error.

B. *False Positive Reactions.*

1. Pseudoagglutination or rouleaux formation. The cells are arranged in rows resembling a stack of coins. Groups of these may have the appearance of agglutination. If examined under the high dry objective of the microscope, each cell retains its normal shape while in true agglutination individual cells are fused with others. A drop of 0.85% NaCl solution may be added and the mixture agitated. The cells disperse if there is rouleaux formation but remain clumped in true agglutination.
2. Agglutination due to "cold agglutinins." These are present in an occasional blood and cause agglutination when the serum and cells are cold or the test is carried out below 20°C.
3. Agglutination due to the presence of "autoagglutinins" in a few individuals. Although the autoagglutinins are in the serum, the cells of some of these individuals will also show agglutination regardless of the testing serum. The test must be performed at 37°C. or the cells must be washed with 0.85% NaCl solution at 37°C. before using in the test. Crossmatching tests must be carried out at body temperature as the serum of such a patient agglutinates cells of all groups at lower temperatures as well as his own cells.
4. The presence of small clumps of cells in the blood cell suspension when it is made from clotted blood, or when the tube is not invert-

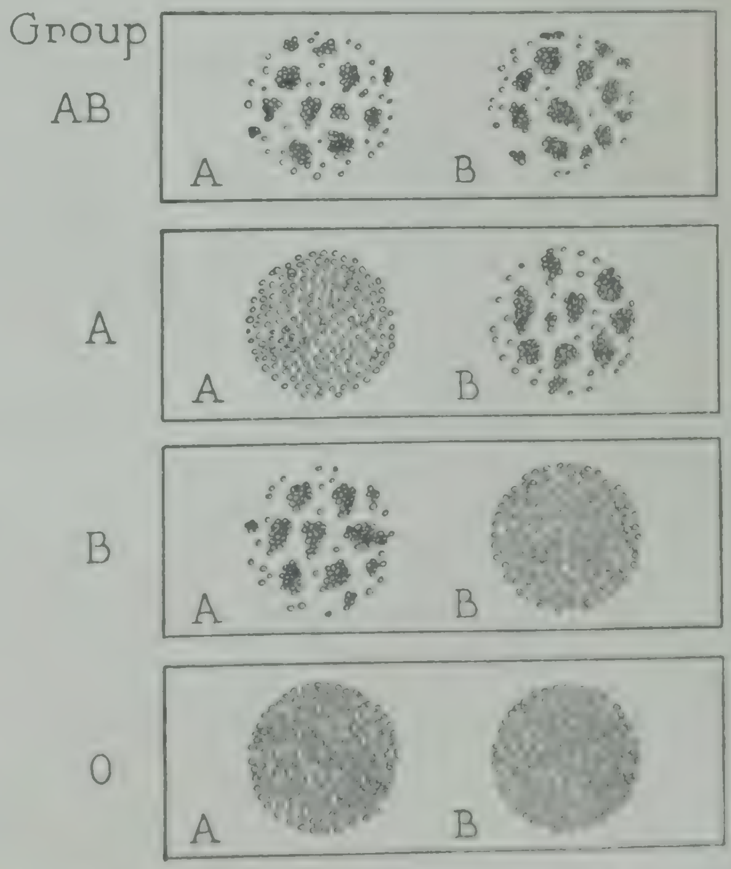


Fig. 26. Reaction of Group A and Group B Typing Serums with the Corpuscles of the Four Major Blood Groups.

ed after adding fresh blood to the saline solution. This can be ruled out by examining a drop of the cell suspension with the low power objective of the microscope.
5. Bacteriogenic agglutination due to changes in either the blood cells or serum produced by certain bacteria; this will not occur if a fresh cell suspension and typing serums are used.
6. Contamination of typing serum with clumps of bacteria, fungi, or molds which may be mistaken for clumped red blood cells.
7. Drying of the preparation.

Cross-Matching

I. General Considerations.

A. *Cross-Matching.*

1. Cross-matching is done to determine compatibility between the blood of the patient and the blood of the prospective donor.
2. Cross-matching must be done before each transfusion.
3. If the patient is receiving a series of transfusions, fresh blood of the patient must be obtained for cross-matching before each new transfusion.
4. Cross-matching done at room temperature will not rule out incompatibility due to Rh agglutinins which act only at body temperature.
5. When plasma or serum transfusions are given, it is only necessary to cross-match the patient's cells with the serum or plasma. However, if pooled serum or plasma from a number of individuals is used, it need not be cross-matched.
6. When a red blood cell suspension (citrated blood which has been centrifuged, the plasma removed, and the cells suspended in 0.85% NaCl solution) is used for a transfusion, it is only necessary to cross-match the patient's serum with the cell suspension.

B. *Donors.*

1. A serology test for syphilis must be done on the donor's serum before each transfusion.
2. A donor of the same group as the patient must be used; only under extreme emergency should a person belonging to group O be used as a universal donor.
3. A person with a history of tuberculosis, malaria, infectious hepatitis, allergy, blood dyscrasia, anemia, or a recent infection or jaundice should not be used as a donor for a transfusion.
4. The donor should not eat for 3 hours before the blood is drawn for the transfusion.

II. Test Tube Method.

A. *Materials Needed.*

1. Blood cell suspension of both patient and donor (see solutions for blood typing).
2. Serum of both patient and donor.
3. Small test tubes (7 mm. in diameter).

B. *Method.*

1. Mark one small test tube P.C. (patient's ce + D.S. (donor's serum).
2. Mark another small test tube P.S. (patie serum) + D.C. (donor's cells).
3. In the tube marked P.C.+D.S., place cc. of the patient's cell suspension and 0.1 of the donor's serum.
4. In the test tube marked P.S.+D.C., place cc. of the patient's serum and 0.1 cc. of donor's cell suspension.
5. Shake both tubes and let stand 5 to 60 m utes in a 37°C. incubator depending on emergency of the transfusion. Incubati rules out incompatibility due to the preser of Rh agglutinins.
6. Centrifuge tubes at medium speed for 1 m ute.
7. Observe any hemolysis in the supernat solution.
8. Shake the tubes gently until cells are d tributed in the solution.
9. Agglutination can be seen macroscopical but for a check always place one drop fr each tube on a slide and examine for clum ing with the low power of the microscop
10. If there is any clumping of the cells or a hemolysis, the bloods are not compatib and another donor must be cross-matche

III. Slide Method.

A. *Materials Needed.*

1. Blood cell suspension of both patient and d nor (see solutions for blood typing).
2. Serum of both patient and donor.
3. Slides with paraffin rings as described for t Kline serology test (p. 239).

B. *Method.*

1. Mark the left hand end of the slide with P. (patient's cells) + D.S. (donor's serum) a the other end P.S. (patient's serum) + D. (donor's cells).
2. To the left hand end, add 1 drop of patien cells and one drop of donor's serum usi separate capillary pipettes.
3. To the right hand end, add 1 drop of patien serum and one drop of donor's cells usi separate capillary pipettes.
4. Carefully rotate the slide on a flat surface f 5 minutes to secure thorough mixing.
5. Examine for agglutination of the cells wi the low power objective of the microscope.
6. Continue to examine at intervals for 30 mi utes, mixing each time by tilting the sl

TABLE 71. CLASSIFICATION OF RH-HR BLOOD TYPES AND SUBTYPES (Modified from Wiener and from Race, Mourant, and McFarlane)

h Blood Types	Reaction with serums			Genes	Reaction with serums			Rh Sub-Types*	Approximate distribution (per cent among Caucasians in New York City)
	Anti-Rh′ (C)*	Anti-Rh″ (E)*	Anti-Rh$_0$ (D)*		Anti-Hr′ (c)*	Anti-Hr″ (e)*	Anti-Hr$_0$ (d)*		
....	—	—	—	r (cde)	+	+	+	rr (cde/cde)	13.0
....	+	—	—	R′ (Cde)	—	+	+	R′R′ (Cde/Cde)	0.01
					+	+	+	R′r (Cde/cde)	1.0
′.........	—	+	—	R″ (cdE)	+	—	+	R″R″ (cdE/cdE)	0.005
					+	+	+	R″r (cdE/cde)	0.5
′Rh″.....	+	+	—	R′ ″ or R^y (CdE)	—	—	+	R_yR_y (CdE/CdE)	**
					—	+	+	R_yR' (CdE/Cde)	**
					+	—	+	$R''R_y$ (cdE/CdE)	**
					+	+	+	R_yr (CdE/cde)	0.01
0.........	—	—	+	R^0 (cDe)	+	+	—	R_0R_0 (cDe/cDe)	2.0
					+	+	+	R_0r (cDe/cde)	
$_1$(Rh$_0'$)...	+	—	+	R^1 (CDe)	—	+	—	R_1R_1 (CDe/CDe)	20.0
					—	+	+	R_1R' (CDe/Cde)	
					+	+	—	R_1R_0 (CDe/cDe)	34.0
					+	+	+	R_1r (CDe/cde)	
$_2$(Rh$_0''$)...	—	+	+	R^2 (cDE)	+	—	—	R_2R_2 (cDE/cDE)	3.0
					+	—	+	R_2R'' (cDE/cdE)	
					+	+	—	R_0R_2 (cDe/cDE)	12.0
					+	+	+	R_2r (cDE/cde)	
$_1$Rh$_2$......	+	+	+	R$^{1\,2}$ or R^Z (CDE)	—	—	—	R_ZR_Z (CDE/CDE)	0.2
					—	—	+	R_ZR_y (CDE/CdE)	**
					—	+	+	R_ZR' (CDE/Cde)	**
					+	—	+	$R''R_Z$ (cdE/CDE)	**
					—	+	—	R_1R_Z (CDe/CDE)	**
					+	—	—	R_2R_Z (cDE/CDE)	**
					+	+	—	R_0R_Z (cDe/CDE)	14.5
					+	+	+	R_Zr (CDE/cde)	

isher's nomenclature.
o cases observed.

back and forth to prevent the settling of the corpuscles in the center.

Cover the slide with the top of a Petri dish when not examining it to prevent evaporation and keep at room temperature.

If there is any clumping of the cells, the bloods are not compatible and another donor must be cross-matched.

V. Sources of Error in Cross-Matching.

Incorrect labeling or pipetting of solutions.

Masking of agglutination by hemolysis.

The use of a too dilute or too concentrated cell suspension.

Clumps in the cell suspension used.

Not allowing the preparation to stand a sufficient length of time for agglutination to take place.

Rh and Hr Factors

I. General Considerations.

Rh Factor.

This is an agglutinogen present in the erythrocytes of about 87 per cent of white people and practically 100 per cent of the American Indians, Chinese, Japanese, Filipinos, Hawaiians, and Australian aborigines.

2. The 3 anti-Rh agglutinins make possible 8 Rh types (see Table 71).

B. *Anti-Rh Agglutinins.*

1. There are 3 anti-Rh agglutinins; anti-Rh$_0$ which agglutinates the cells of 85 per cent of the white population, anti-Rh′ agglutinating 70 per cent, and anti-Rh″ agglutinating 30 per cent. These usually occur in the following combinations: anti-Rh$_0'$ (anti-Rh$_0$Rh′) which agglutinates 87 per cent of Rh-positive cells, and anti-Rh$_0''$ (anti-Rh$_0$Rh″) which due to the Rh″ type being very rare agglutinates approximately the same number as anti-Rh$_0$.
2. Rh agglutinins are not normally present in serum and when present are always due to immunization.
3. The Rh agglutinogen acts as an antigen pro-

ducing anti-Rh agglutinins under certain conditions.

  a. An Rh-negative woman carrying an Rh-positive fetus may produce anti-Rh agglutinins which in turn react with the Rh agglutinogen in the fetus resulting in erythroblastosis in the baby.
    1) The best time to test for anti-Rh agglutinins in the mother's serum is from 8 to 20 days following childbirth.
    2) The antibodies are also present in the mother's milk.
  b. An Rh-positive mother may give birth to a baby with erythroblastosis due to Rh incompatibility; that is, she is a different Rh type from that of her husband and child.
  c. An Rh-negative person receiving several transfusions of Rh-positive blood will develop anti-Rh antibodies and further transfusions with Rh-positive blood will result in severe transfusion reactions.

C. *Hr Factor.*

1. The symbol Hr was selected to indicate that the factor is the opposite of Rh because the Hr agglutinogen is present in all rh (Rh-negative) bloods.
2. It has been found that this factor has a reciprocal relationship to the Rh factor in the same manner as agglutinogen M is related to agglutinogen N.
3. There are 3 Hr factors, Hr′ which is related to Rh′, Hr″ which is related to Rh″, and Hr$_0$ which is related to Rh$_0$. (An anti-Hr$_0$ serum has not been discovered with certainty.)
4. The Hr′ factor is found in 80% of the white population, the Hr″ in 97%, and the Hr$_0$ in 63%.
5. These Hr factors make it possible to theoretically subdivide the Rh types into subtypes thus making 27 Rh-Hr blood types (see Table 71).
6. An Hr-negative mother (who would be Rh-positive) may give birth to an erythroblastotic baby (Hr-positive) from immunization to the Hr factor in the fetus, since the mother would have anti-Hr agglutinins in her serum.
7. An Hr-negative person may develop anti-Hr agglutinins from transfusions with Hr-positive blood.
8. Anti-Hr serum only clumps the cells of heterozygous Rh-positive persons thus making all Rh-positive, Hr-negative people homozygous for the Rh factor.

II. Test for Rh Factor.

A. *Typing Serum.*

1. Human anti-Rh typing serums may be obtained from 3 sources but must have a antibody titer.
  a. Rh-negative mothers of children with erythroblastosis.
  b. Rh-negative persons who have rec several transfusions of Rh-positive bl
  c. Rh-negative persons who are willi become immunized by injections of positive cells.
2. Only serums of persons of group AB ca used for Rh typing of all 4 major gr without absorption of anti-A or anti- both agglutinins, because AB serum does contain either of these agglutinins.
3. Serums belonging to the other 3 groups have the anti-A and anti-B agglutinins pre absorbed or they can only be used for typing of their own group.
4. The anti-A and anti-B agglutinins can be sorbed by using the purified A and B stances of Witebsky.
5. For typing patients it is preferable to use a Rh$_0$ serum which will agglutinate 85 per of cells, although it will record all Rh′ Rh″ people, who lack Rh$_0$, as Rh-negativ
  a. If persons of group Rh′ or Rh″, who Rh$_0$, are to receive a transfusion, it is they be transfused with Rh-negative blo
  b. If transfused with Rh-positive blood would most likely be of group Rh$_0$ the person might develop anti-Rh$_0$ glutinins.
6. All blood donors should be typed with a Rh$_0$′ serum which will agglutinate 87 cent of cells; this will eliminate giving negative people a transfusion of group blood. (Type Rh″ is so rare that it is regarded.)
7. Typing serums containing agglutinating saline antibodies can be used only for the tube method.
8. Typing serums containing blocking a bodies can be used only for the slide meth
9. Hetero-immune serums from guinea treated with blood from Rhesus monkeys not satisfactory, because they agglutinate blood of all infants regardless of the Rh ty

B. *Test Tube Method.*

1. Place 1 drop of anti-Rh typing serum in small test tube (75 mm. long and 7 mm. side diameter).
2. Add 1 drop of a 2% saline suspension the red blood cells to be tested.
3. Shake tube and place in an incubator water bath at 37°C. for 1 hour.
4. Examine the sediment over a concave mirr
  a. If negative, the cells settle into the botto

of the tube in a round, compact, circumscribed mass having a sharp crisp edge.

b. If positive, the cells spread over a large area of the bottom of the tube in a mass having a granular or slightly irregular margin and often exhibits several slightly crinkled areas in the sediment.

Agitate *gently;* the positive tests will show flocculation.

If no flocculation is visible, centrifuge for one minute at about 500 revolutions per minute.

Tilt the tube gently about 6 times to loosen some of the sediment, place a drop on a slide, and examine with the low power objective of the microscope.

Any definite clumping, even if the clumps contain only 3 or 4 cells, is evidence the person is Rh-positive.

Slide Method.

Place 1 drop of anti-Rh blocking serum of high titer on a slightly warmed slide and add 1 drop of fresh, unclotted or oxalated whole blood (do not use a saline suspension).

Keep the slide warmed to 37°C. (avoid excessive heating) and tilt it from side to side to permit intermixture of cells and serum.

Examine for agglutination by holding the slide on a piece of ground glass placed over an electric light bulb.

If the cells are Rh-positive, a finely granular clumping usually becomes visible in 15-20 seconds and in less than 1 minute the mixture is converted into large sticky clumps. It may take longer than 1 minute if the slide and typing serum are not at 37°C. at the beginning of the test.

If this test is negative, it should be checked by the test tube method which is more sensitive.

Sources of Error.

Use of typing serum that is contaminated or so old that it has lost its potency.

Use of incorrect temperature in performing the test.

Too light or too heavy a cell suspension.

Clumps in the cell suspension.

Too rough handling of specimen following incubation.

Confusion of rouleaux formation with true agglutination.

Bacterial contamination or hemolysis of the blood specimen.

III. Test for Anti-Rh Agglutinins.

General Considerations.

There are 2 types of anti-Rh agglutinins.

a. The agglutinating antibody which agglutinates Rh-positive erythrocytes suspended in saline solution.

b. The blocking antibody which unites with Rh-positive erythrocytes in saline suspensions, but does not cause agglutination unless the cells are suspended in albumin or plasma. These have been called incomplete, partial, inhibiting, coating, glutinin, thermostable, mature, late, and hyperimmune antibodies by different investigators.

c. The Coombs' test using absorbed rabbit anti-human-globulin serum may be used to detect blocking antibodies in the mother's serum or in the baby's blood. Methods come with the commercial Coombs' serum.

d. Both agglutinating and blocking antibodies may be present in the same serum.

2. Anti-Rh antibodies are most active at 37°C. and are called warm agglutinins.
3. Both types act as hemolysins within the body, although they do not produce hemolysis when exposed to Rh-positive cells in the test tube.
4. Agglutinating antibodies are rarely found in the blood of infants, but blocking antibodies are found if present in the blood of the mother.
5. The presence of blocking antibodies is more serious than agglutinating antibodies; about 85 per cent of mothers having stillborn infants have blocking antibodies.

B. *Patient's Serum.*

1. Separate the serum from 10 cc. of blood.
2. Inactivate in a 56°C. water bath for 20 minutes.

C. *Slide Method of Diamond and Abelson.*

1. Place 0.2 cc. of fresh, oxalated, whole blood of the following types on a slide.
    a. Group O, rh (Rh-negative).
    b. Group O, Rh_1.
    c. Group O, Rh_2.
    d. If both Rh_1 and Rh_2 bloods are not available, from 6 to 8 group O, Rh_0 bloods should be used.
2. Add 0.1 cc. of the inactivated serum to be tested to each drop of blood.
3. Rotate or tilt the slide gently for 1 to 3 minutes; keeping the slide warmed to 37°C.
4. Examine for agglutination by holding the slide on a piece of ground glass placed over an electric light bulb.
5. If the patient's serum contains agglutinating or blocking antibodies, or both, one or more of the Rh-positive bloods will show agglutination while the Rh-negative control will not show agglutination.

D. *Agglutination Test for Agglutinating (Saline) Antibodies.*

1. Obtain fresh, oxalated blood of the following types:
    a. Group O, rh (Rh-negative).

   c. Group O, Rh_2.
   b. Group O, Rh_1.
   d. If both Rh_1 and Rh_2 bloods are not available, 6 to 8 group O, Rh_0 bloods should be used.
2. Wash the cells of each of the above types once with 0.85% NaCl solution. (Not absolutely necessary.)
3. Make an approximately 2% suspension of the erythrocytes in 0.85% NaCl solution of each type of blood.
4. Makes serial dilutions of the patient's inactivated serum, 1-5, 1-10, 1-20, and 1-40 dilutions.
5. Set up a row of 5 tubes for each cell suspension made above.
6. Place 1 drop of undiluted serum in the first tube of each row, 1 drop of the 1-5 dilution to the second tube of each row, one drop of the 1-10 dilution to the third tube of each row, 1 drop of the 1-20 dilution to the fourth tube of each row, and 1 drop of the 1-40 dilution to the last tubes.
7. Add 1 drop of the Rh_1 cell suspension to each tube in the first row.
8. Add 1 drop of the Rh_2 cell suspension to each tube in the second row.
9. Add 1 drop of the rh cell suspension to each tube in the third row.
10. Shake the tubes and place in a water bath at 37°C. for 1 hour.
11. Read in the same manner as for Rh agglutinins.
12. If agglutination occurs, the titer of the agglutinating antibodies is reported according to specificity.
   a. If only Rh_1 cells are agglutinated, the agglutinin is anti-Rh'. Such serums should be tested for anti-Rh_0 blocking antibodies.
   b. If only Rh_2 cells are agglutinated, the agglutinin is anti-Rh''. Such serums should be tested for anti-Rh_0 blocking antibodies.
   c. If both the Rh_1 and Rh_2 cells but not type rh cells are agglutinated, the agglutinin may be anti-Rh_0, anti-Rh_0', or anti-Rh_0''.
      1) Mix 5 parts of the patient's serum with 1 part of a potent anti-Rh_0 blocking serum and repeat the titration.
      2) If there is no agglutination with any of the cells, it is anti-Rh_0.
      3) If there is agglutination only with Rh_1 cells, it is anti-Rh_0'.
      4) If there is agglutination only with Rh_2 cells, it is anti-Rh_0''.

E. *Method I for Blocking Antibodies (C glutination Test).*

1. Make a 2% suspension of each type of bl listed under agglutination test in a 20% tion of bovine albumin.
   a. A vial containing 20 cc. of 30% alb solution from bovine plasma for anti- conglutination tests may be obtained fro Armour Laboratories, 142 W. 42nd S Chicago, Ill.
   b. Make a 20% solution by adding 10 cc. sterile 0.85% NaCl solution to the 20 c of 30% bovine albumin.
2. Place one drop of each suspension of eryt rocytes in separate test tubes.
3. Add 1 drop of the patient's inactivated seru to each test tube.
4. Shake the rack and tubes vigorously and the place in a 37°C. water bath for 1 hour.
5. Shake the tubes gently, though more strong than in the agglutination test, and read th reactions.
6. If there is no agglutination of the cells in an of the tubes, the conglutination test is neg ative.
7. If there is any agglutination with the Rh pos tive cells, the test must be repeated using a 1- 1-10, 1-20, and 1-40 dilution of serum mad with the 20% bovine albumin solution.
8. These dilutions must be set up with rh cell (Rh-negative) as well as the Rh positive cells
9. The greatest dilution of serum showing ag glutination is the titer of antibodies.

F. *Method II for Blocking Antibodies (Block ing Test).*

1. It is only necessary to do this test when th agglutination test is negative.
2. The test is a continuation of the agglutinatio test.
3. Add 1 drop of a suitably diluted (to titer o 10 units) active anti-Rh_0 agglutinating serum to each tube of the first 2 rows (containing Rh-positive cells) of the agglutination test.
4. Shake the tubes, incubate in a 37°C. wate bath for 1 hour, and read as in the agglutination test.
5. The results are reported as follows:
   a. Blocking test negative when all the tubes containing Rh-positive cells show strong agglutination.
   b. Blocking test doubtful when all the tubes containing Rh-positive cells show agglutination, but the clumping is weak in the tubes containing the highest concentrations of the patient's serum.
   c. Blocking test is positive when the highest dilutions of the patient's serum show complete or almost complete inhibition of the

reactions of the anti-Rh_0 agglutinating serum.

edicolegal Use of Blood Groups or Grouping

lood grouping in medicolegal cases hould be done only by persons especially killed in the technique and fully familiar with he principles involved.

. Exclusion of Paternity or Maternity.

lood groups can only be used to exclude lleged paternity or maternity.

lood agglutinogens are transmitted as Menelian dominants; therefore, blood groups are nherited according to Tables 72 and 73. s many types of a blood as possible should e determined because each new independent gglutinogen doubles the number of subdiviions of human blood, and therefore gives a reater chance of ruling out alleged paternity r maternity.

. The agglutinogens A_1, A_2, B, M, N, P, Rh_0, Rh′, and Rh″ and the secretion or nonsecretion in saliva of group-specific substances make possible 576 distinct types of individuals.

. The Hr groups make possible even more subdivisions.

ABLE 72. HEREDITY OF THE LANDSTEINER BLOOD GROUPS (Bernstein's Theory)

Groups of parents	Groups of children possible	Groups of children not possible
O + O	O	A, B, AB
O + A	O, A	B, AB
O + B	O, B	A, AB
A + A	O, A	B, AB
A + B	O, A, B, AB	—
B + B	O, B	A, AB
O + AB	A, B	O, AB
A + AB	A, B, AB	O
B + AB	A, B, AB	O
AB + AB	A, B, AB	O

LE 73. HEREDITY OF THE M, N, AND MN BLOOD GROUPS

pes of parents	Per cent children of types		
	M	N	MN
M + M	100	0	0
M + N	0	0	100
M + MN	50	0	50
N + N	0	100	0
N + MN	0	50	50
MN + MN	25	25	50

roup-specific Substances.

rouping human beings into 4 major groups not limited to the blood, since group-specific substances are present in the cells of the organs, in the body fluids, and in the secretions.

2. These group-specific substances are present in a lipoidal and in a water-soluble form.
3. The lipoidal form is present in all human beings, but only 80 per cent have the water-soluble form which is the form secreted in saliva and gastric juice.
4. The presence or absence of the water-soluble group-specific substances in saliva divides human beings into 2 more groups, secretors (S) and nonsecretors (s).
5. The presence of group-specific substances can be determined by either of the following methods.
   a. The inhibition of agglutination of group A blood cells by anti-A serum or of group B blood cells by anti-B serum.
   b. Precipitin tests using potent anti-A and anti-B immune rabbit serums.

III. Exclusion of Mix-Up of Infants.

A. The determination of the blood groups of the infants and parents can usually determine whether there has been a mix-up of infants in the hospital.

B. See Tables 72 and 73 for the groups of children from different combinations of groups of the parents.

IV. Blood Grouping in Criminal Cases.

A. *Determination of Blood Group from Blood Stains.*

1. To exclude the possibility that the blood found on a suspect is his own blood and not that of the victim.
2. To exclude the possibility that the blood found on the victim is his own blood and not that of the suspect.

B. *Seminal Fluid.*

1. The group-specific substances can be determined in seminal fluid.
2. This evidence can be used only in the exclusion of a suspected person in rape cases.

C. *Saliva.*

1. The group-specific substances can be determined in saliva. However, one must also know whether the suspected person is a secretor or nonsecretor.
2. Traces of saliva on gummed edges of envelopes and on cigarette stubs may be typed.

Demonstration of Cold Agglutinins

Test for Cold Agglutinins.

A. *Materials Required.*

1. Patient's serum obtained from fresh blood

that has not been placed in the refrigerator to clot.

2. *Human Erythrocyte Suspension—2%.*
    a. Obtain human blood belonging to group O and place in a bottle containing an anticoagulant.
    b. Wash 3 times with 0.85% NaCl solution.
    c. Pack the cells and make a 2% suspension in 0.85% NaCl solution.
    d. Cells of the patient can be used.

B. *Method* (See Table 74).

1. Set up a series of 12 small test tubes in a rack and place 1.5 cc. of 0.85% NaCl solution in the first tube and 1 cc. in each of the following tubes.
2. Add 0.5 cc. of the patient's serum to the first tube and mix.

TABLE 74. TEST FOR COLD AGGLUTININS

Tube	0.85% NaCl solution in cc.	Serum in cc.	Dilution of serum	Erythrocyte suspension (2%) in cc.
1	1.5	0.5	1-4	0.1
2	1	1 of 1-4	1-8	0.1
3	1	1 of 1-8	1-16	0.1
4	1	1 of 1-16	1-32	0.1
5	1	1 of 1-32	1-64	0.1
6	1	1 of 1-64	1-128	0.1
7	1	1 of 1-128	1-256	0.1
8	1	1 of 1-256	1-512	0.1
9	1	1 of 1-512	1-1024	0.1
10	1	1 of 1-1024	1-2048	0.1
11	1	1 of 1-2048*	1-4096	0.1
Control 12	1			0.1

*Discard 1 cc. from tube 11.

3. Transfer 1 cc. from the first tube to th second tube and mix.
4. Transfer 1 cc. from the second tube to th third tube and so on through the elevent tube from which 1 cc. is discarded.
5. Add 0.1 cc. of a 2% suspension of washe human erythrocytes belonging to Group to each tube.
6. Mix and place in the refrigerator at 0 t 4°C. overnight.
7. Read immediately after taking out of the re frigerator after inverting each tube agains the finger 3 times.

    ++++ = firm disk-shaped mass of sedimen which does not break up when the tub is inverted.
    +++ = disk easily broken into large flakes, th fluid is clear.
    ++ = fine agglutination, fluid not transparen
    + = barely perceptible, but definite aggluti nation when viewed through the lo power objective of the microscope.
    0 = no agglutination.

8. If positive, allow tubes to stand at roo temperature for several hours and then rea again to ascertain whether the reaction i reversible at room temperature and therefor a true cold agglutination.

C. *Interpretation.*

1. A titer of 1-32 or higher is suggestive o virus pneumonia.
2. High titers are also obtained in Africa trypanosomiasis, leishmaniasis, hemolytic anemias, mumps orchitis, and blackwater fever

Clinical Chemistry

General Instructions

I. Collection of Blood.

eneral Considerations.

he patient must not have any food for 12 ours before blood is drawn for most chemi- l determinations. For determinations not sing fasting blood, see footnote of Table 75.

he amount and type of blood (oxalated or otted) necessary for each determination ill be found in Table 75.

scorbic acid, CO_2, chloride, sugar, cho- sterol esters, and all enzyme determinations ust be made within one-half hour after the ood is drawn. If fluoride and thymol are ed as preservatives, blood for sugar de- rminations may, if necessary, be kept for 6 ys without alteration of its sugar content. ll other determinations should be made the me day that the blood is obtained.

If determinations on oxalated blood can not be made the same day as the blood is obtained, the protein-free filtrate should be made, 1 drop of toluene added, and kept in the refrigerator.

If determinations on serum can not be made immediately, the serum must be separated and stored in the refrigerator.

enous Blood.

ollow directions under collection of venous lood in the Section on Hematology, page 3. The syringe and needle must be dry prevent hemolysis.

lood for serum determinations is placed in ry chemically clean test tubes and allowed clot.

lood for whole blood or plasma determina- ons is placed in 1 ounce bottles containing anticoagulant of the proper kind and nount as given in Table 76.

apillary Blood for Micromethods.

Iand must be warm with good circulation.

cold, have patient immerse the hand in ot water and rub briskly with a towel.

leanse tip of patient's finger with alcohol nd dry.

rasp patient's finger firmly near the hand and push along the skin to finger tip and hold tightly.

4. With a blood lancet of the spring release type with a blade at least 3/16 of an inch long which has been dipped in alcohol, puncture the tip of the finger across the lines (blade crosswise to the length of the finger). If the prick does not bleed freely, puncture again so the second prick adjoins the first making a larger cut.
5. Have patient hold arm and hand down so that the blood drops into a 1 cc. glass vial containing an anticoagulant. If blood does not flow easily, massage finger gently lengthwise at intervals to increase flow of blood. Do not squeeze the finger tip.
6. Stir blood thoroughly with a toothpick or pin between each 2 drops.
7. Fill vial at least two-thirds full of blood.

II. Anticoagulants (See Table 76).

A. *Preparation of Bottles Containing Anticoagulant.*

1. If a solution of anticoagulant is used, pipette the proper amount of solution according to Table 76 into a chemically clean bottle and heat to 60 or 70°C. until the water has evaporated and the dry chemical is left distributed on the bottom of the bottle. Do not heat over 70°C. or leave bottles in contact with the heat too long, because the oxalate is converted into carbonate.
2. Paraffined corks or rubber stoppers are used in the bottles.

B. *Action of Anticoagulants.*

1. The chemical anticoagulants prevent blood from clotting by the removal of ionized calcium.
2. Heparin (antithrombin) is thought to prevent clotting by the inactivation of prothrombin.

III. Glassware.

A. *Beakers, Flasks, etc.*

1. Should be of pyrex quality.
2. Must be chemically clean and dry.

TABLE 75. QUANTITY AND TYPE OF BLOOD AND NORMAL VALUES FOR CHEMISTRY DETERMINATIONS

	Normal Value Mg. per 100 cc.
Whole Blood**	
Alcohol (3 cc.)	None
Nonprotein N (5 cc.) (no clots)	25—35
Pyruvic Acid (2 cc.)	0.6—1.2
Sugar (3 or 0.5 cc.) (no hemolysis)	80—120
Urea N (5 cc.)	10—15
Plasma	
Ascorbic Acid (2 cc.) (no hemolysis)	Over 0.6
Carotene (15 cc.)	100—300 I. U.
Fibrinogen (5 cc.)	200—400
Vitamin A (15 cc.)	100—250 I.U.
Serum	
Albumin (10 cc.) (no hemolysis)	3.5—5.6 gm.
Albumin-Globulin ratio	1.5:1 to 3:1
Amylase (10 cc.)	80—150
*Bromides (7 cc.)	None
Calcium (15 cc.) (no hemolysis)	
Adults	9.5—11.0
Children	10.0—11.5
Infants	10.5—12.0
CO_2 Capacity (10 cc.) (no hemolysis)	
Adults	53—70 vol. %
Infants	40—55 vol. %
*Cephalin Flocculation (5 cc.)	None
Chlorides (NaCl) (7 cc.) (no hemolysis)	580—630
Cholesterol (5 cc.)	
Men	150—240
Women	180—260
Serum Continued	
Cholesterol Esters (7 cc.) (no hemolysis)	70—75% of total
Creatinine (7 cc.)	0.8—2
Globulin	1.3—3.2 gm.
Icterus Index (5 cc.) (no hemolysis)	4—6 units
Lipase (7 cc.)	0—0.3 cc. of 0.05 N Na
Lipochromic Index (10 cc.) (carotene)	0—0.1
pH (no hemolysis) (10 cc.)	7.35—7.45
Phosphorus (5 cc.) (no hemolysis)	
Adults	2.5—4
Children	4.5—5.5
Infants	5.5—6.5
Alkaline Phosphatase (7 cc.) (no hemolysis)	
Adults	1.5—4 units
Children	5—12 units
Acid Phosphatase (7 cc.)	0—3.25 units
Potassium (10 cc.) (no hemolysis)	18—21
Salicylic Acid (10 cc.)	None
Sodium (10 cc.)	325—350
*Sulfonamides (7 cc.)	None
*Thiocyanate (15 cc.)	None
Thymol Turbidity (5 cc.)	0—5 units
Flocculation	None
Total Protein (7 cc.) (no hemolysis)	6—8 gm.
Uric Acid (7 cc.)	2—5
Van den Bergh (7 cc.)	0.2—0.8

*Blood can be obtained after a meal, all other determinations must be made on fasting blood.
**Ten cc. of whole oxalated blood is sufficient for any combination of tests with whole blood.

B. *Pipettes.*

1. Pipettes must be chemically clean and dry.
2. Good quality pipettes must be used—Exax Blue Line quality is considered reliable.
3. Normax precision quality should be used for making calibration curves on a photoelectric colorimeter.
4. *Types of Pipettes.*
    a. Transfer or volumetric pipettes are used when accurate measurement is wanted as in measuring filtrates, standards, and most reagents. They are calibrated "to deliver" a fixed volume so the residue remaining in the tip is not blown out.
    b. Ostwald-Folin pipettes are used when pipetting blood or viscid materials. They are calibrated "to contain" a fixed quantity so the last few drops remaining in the delivery tip are blown out.
    c. Mohr pipettes of Exax quality are used

TABLE 76. BLOOD ANTICOAGULANTS[1]

nical	Requirement for 10 cc. of blood	Advantages and disadvantages
n (best)	1 cc. of a 1% solution or 10 mg.	More soluble than sodium or potassium oxalate. Prevents a white precipitate in uric acid determinations.
um	0.1 cc. of a 20% solution or 20 mg.	Concentration of 3 mg. or more per cc. alters the electrolyte distribution in blood; interferes with precipitation of proteins (Folin-Wu method); gives too low sugar values; and may cause clouding when Nesslerizing.
n e	1 cc. of a 2% solution or 20 mg.	Same as potassium oxalate.
n e	Grind 10 parts sodium fluoride, 1 part thymol, 3 parts lithium oxalate together. Used as a powder, 35 mg. for 10 cc. or 4 mg. for 1 cc. of blood.	Used in microsugar determinations, will preserve the blood for 24 hours at room temperature and 5 or 6 days at refrigerator temperature. Interferes with action of urease in urea determinations. If samples of blood are to be mailed, use 10 mg. per 1 cc. of blood.
m	Used as powder, 30 mg.	Impractical for routine use; employed only when determining mineral constituents of whole blood.
n	2 cc. of a 3% solution or 60 mg.	Alters the composition of blood; prevents clotting only a few hours; interferes with many chemical determinations.
in	Fill dead space of syringe with a 1% solution (Liquaemin made by Organon) or add a few drops to the blood (2 mg. necessary).	Expensive; the dry powder is not readily soluble in blood.
onium otassium e mix-	1 cc. of a solution containing 1.2% ammonium oxalate and 0.8% potassium oxalate.	Used only for hematology work. Carefully adhere to the exact proportions of blood to oxalate.

preparation and action of anticoagulants see page 255.

when it is not important to have an exact quantity of a reagent. Normax quality of Mohr pipettes for more exacting work are used only when a fraction of a cc. is required.

lethod of Using Pipettes.

. Draw solution to be pipetted slightly above the mark.

. Remove the fluid adhering to the outside of the pipette with a clean cloth or cleansing tissue.

. Let the fluid out to the mark.

. Hold the pipette almost vertical, the tip against the inner wall of the receiving vessel.

. Unless Normax quality pipettes are used, the rate of delivery should be controlled with the finger over the upper opening.

alibration of Pipettes.

. Pipette must be thoroughly cleaned in cleaning solution.

. Allow a sample of distilled water (previously boiled) in a clean beaker to attain the temperature of the balance room.

. Weigh a weighing bottle or beaker containing paraffin oil a few mm. thick to prevent evaporation and record the weight.

d. Suck up distilled water into the pipette several times so that the pipette is the same temperature as the water.

e. Record the temperature of the water.

f. Fill the pipette to the mark with the distilled water as described under method of using pipettes, and holding the pipette in a vertical position allow the water to flow into the weighed bottle or beaker.

g. Allow the pipette to drain 15 seconds and touch the tip to the side of the beaker.

h. Weigh the bottle and calculate the volume of water according to the weight and temperature.

Temp. C°.	Wt. of 1 cc.	Vol. of 1 gm.
15	0.9979	1.0021
16	0.9978	1.0022
17	0.9977	1.0023
18	0.9975	1.0025
19	0.9973	1.0027
20	0.9972	1.0028
21	0.9970	1.0030
22	0.9968	1.0032
23	0.9966	1.0034
24	0.9964	1.0036
25	0.9961	1.0039
26	0.9959	1.0041
27	0.9956	1.0044
28	0.9954	1.0046
29	0.9951	1.0049
30	0.9948	1.0052

- i. If the original mark is not accurate, make another mark with a wax pencil and test the pipette again.
- j. Continue until the mark is located correctly; repeated results must check within 0.004 cc. for a 2 cc. pipette, 0.01 cc. for a 5 cc. pipette, and 0.02 cc. for a 10 cc. pipette (error of 0.002 cc. for each cc.).
- k. Etch the line or mark with a file.
- l. One may calculate where a line should be by weighing water delivered from two marks a definite distance apart; such as 50 mm.

  Example:
  - 1) Weight of water at 20°C. delivered from a 10 cc. pipette from 2 marks 50 mm. apart are 9.900 and 10.275 gm., respectively.
  - 2) Weight of column of water between marks is 0.375 gm., or $\frac{0.375}{50} = 0.0075$ gm. per mm. of column length.
  - 3) Ten cc. of water at 20°C. should weigh 9.972 gm. or 0.072 gm. more than that delivered from the lower mark.
  - 4) The correct mark is $\frac{0.072}{0.0075}$ or 9.6 mm. above the lower mark.

C. *Cleaning Glassware.*

1. Beakers, flasks, funnels, etc., must be cleaned by washing with soap and water as soon as possible after being used.
2. Pipettes should be put in a jar containing water immediately after being used.
3. After the glassware and pipettes are thoroughly washed, they are placed in cleaning solution for 4 to 24 hours.
4. After removing from cleaning solution, the glassware and pipettes must be washed 8 times with tap water and 4 times with distilled water.
5. They are allowed to dry either by draining or heating in a warming oven.
6. *Preparation of Cleaning Solution:*
   - a. Dissolve 100 gm. of sodium dichromate (commercial) in 200 cc. of water with the aid of heat.
   - b. Add 800 cc. of commercial sulfuric acid, keeping the container cool by setting it in cold water.

IV. Solutions.

A. *General Considerations.*

1. Distilled water is always used in chemical procedures and for making up solutions.
2. Cool all solutions to 20°C. before diluting to volume in volumetric work and mix th oughly before using by inverting flasks times.

B. ***A standard solution*** is one in which the ex concentration of the solute is known.

C. ***A molar solution*** is one in which one lit of the solution contains the number of gra of the solute equal to its molecular weig (see Table 77 or 78).

1. For example: M HCl = 36.46 gm. per lit and M H_2SO_4 = 98.08 gm. per liter.
2. Molar solutions may be used in multiple i.e., 2 M, or fractions, i.e., M/15 or 1/15 M

D. ***A normal solution*** contains in a liter of solu tion one gram-equivalent weight of solute A gram-equivalent weight has either on gram atom of reactive hydrogen, or i capable of replacing or neutralizing tha quantity (see Table 77 or 78).

1. In determining the unknown strength of solution (A), its normality (N) multiplied by the volume used in the titration is equa to the normality (N) of the standard solution (B) multiplied by the volume used in the titration.

   Example: N. of A × cc. of A used = N. of B × cc. of B used.
2. Normal solutions may be used in multiples or fractions of normal, such as 2 N, 2.5 N, 0.1 N (decinormal or N/10), 0.05 N (twentieth or N/20), or 0.02 N (fiftieth or N/50).
3. Directions for making the most frequently used normal solutions will be found in the Section on Solutions Used in Routine Tests, page 357.

E. ***A physiologic solution*** is an isotonic solution of crystalloids in water resembling the crystalloids in normal blood serum. (An isotonic solution is one having the same osmotic pressure as normal blood serum.)

1. *Physiologic Saline.*

Sodium chloride	8.5 gm.

Distilled water to make 1 liter.

2. *Ringer's Solution.*

Sodium chloride	7.000 gm.
Calcium chloride	0.026 gm.
Potassium chloride	0.350 gm.

Distilled water to make 1 liter.

3. *Locke's Solution.*

Sodium chloride	9.00 gm.
Calcium chloride	0.24 gm.
Potassium chloride	0.42 gm.
Sodium carbonate	0.20 gm.
Glucose	2.50 gm.

Distilled water to make 1 liter.

77. ATOMIC WEIGHTS COMMONLY USED

	(Ba)	137.36	Manganese	(Mn)	54.93
ne	(Br)	79.916	Mercury	(Hg)	200.61
n	(Ca)	40.08	Molybdenum	(Mo)	95.95
	(C)	12.01	Nitrogen	(N)	14.008
ne	(Cl)	35.457	Oxygen	(O)	16.000
	(Co)	58.94	Phosphorus	(P)	30.980
r	(Cu)	63.57	Potassium	(K)	39.096
	(Au)	197.2	Silver	(Ag)	107.880
gen	(H)	1.008	Sodium	(Na)	22.997
	(I)	126.92	Sulfur	(S)	32.06
	(Fe)	55.85	Tin	(Sn)	118.70
	(Pb)	207.21	Tungsten	(W)	183.92
m	(Li)	6.940	Uranium	(U)	238.07
sium	(Mg)	24.32	Zinc	(Zn)	65.38

TABLE 78. NORMAL SOLUTIONS

Reagent*	Molecular weight	Grams in one liter	Approximate cc. per liter
lrochloric (HCl), sp.gr. 19, 37%	36.47	36.47	83.0**
lic ($H_2C_2O_4 \cdot 2\,H_2O$)	126.07	63.035	
uric (H_2SO_4), sp.gr. 84, 95.5%	98.07	49.035	28.0
xides†			
assium (KOH)	56.10	56.10	
ium (NaOH)	40.01	40.01	
assium permanganate ($KMnO_4$)	158.03	31.61***	
assium thiocyanate (KSCN)	97.17	97.17	
er nitrate ($AgNO_3$)	169.89	169.89	
ium carbonate (Na_2CO_3)	106.00	53.00	
ium chloride (NaCl)	58.45	58.45	
ium oxalate ($Na_2C_2O_4$)	134.01	67.005	
ium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)	248.20	248.20	

ls of chemicals must be carefully checked to see if are anhydrous or contain water of crystallization increases their molecular weight.

cc. per liter is obtained by dividing the equivalent t by the product of the specific gravity times the per f the solution.

acid solutions potassium permanganate is reduced to of potassium and manganous oxides with loss of n; the latter is taken up by the substance oxidized:

$$2\,KMnO_4 \rightarrow K_2O + 2\,MnO + 5\,O^{=}$$

fore, 2 gram-molecules of $KMnO_4$ correspond to m-atoms of oxygen, or 10 gram-atoms of hydrogen. gram-molecule of $KMnO_4$ corresponds to 5 gram- of hydrogen so a normal solution contains 1/5 of the ular weight.

nal solutions of hydroxides are made from saturated ons. The hydroxides are hygroscopic and combine carbon dioxide of the air to form carbonate so that mpossible to weigh them accurately.

uffer Solutions are solutions which are apable of maintaining their hydrogen ion oncentrations in spite of the addition of ppreciable quantities of acid or alkali.

lix solutions according to Table 79 for different pH, these will keep in a pyrex glass bottle in the refrigerator for several weeks.

2. *M/15 Potassium Acid Phosphate.*

KH_2PO_4 ("Sørensen salt") .... 9.08 gm.
(or 9.274 gm. of $NaH_2PO_4 \cdot H_2O$).
Distilled water to make 1 liter.

3. *M/15 Disodium Phosphate.*

Na_2HPO_4 (anhydrous) Merck .... 9.47 gm.
(or 11.87 gm. of $Na_2HPO_4 \cdot 2H_2O$ "Sørensen salt").
Distilled water to make 1 liter.

4. *M/10 Potassium Acid Phthalate.*

$KHC_8H_4O_4$ .... 20.418 gm.
Distilled water to make 1 liter.

V. Chemicals.

A. *Quality.*

1. Reagent or chemically pure (c.p.) quality is used in all chemical determinations unless otherwise indicated.
2. Commercial or technical and U.S.P. quality can be used only when definitely specified.

B. *Types.*

1. Labels must be read carefully to note whether the chemical is anhydrous or crystalline.
2. The crystalline form contains water of crystallization and therefore has a greater molecular weight than the anhydrous form.
3. Inorganic chemicals used for standards should be dried in a 110°C. oven for 1 hour and then placed in a desiccator over sulfuric acid or calcium chloride overnight before weighing on a delicate chemical balance. Organic chemicals should not be heated but placed in a desiccator for 2 days before weighing.

VI. Analytical Balance.

A. *General Considerations.*

1. The balance must be level.
2. The release and arrest of the beam and pans.
   a. Release the beam before releasing the pans.
   b. Release and arrest the beam with a slow steady movement, avoiding jerky movement which injures the knife-edge.
   c. The beam should be arrested only when in a horizontal position.
3. Never place an object, not even the smallest weight, upon the pan or remove one from it unless the pans are supported.
4. Always place the weights and objects to be weighed in the middle of the pans.
5. Handle all the weights with the tweezers provided for this purpose and never use these tweezers for any other purpose.

TABLE 79. COMPOSITION OF BUFFER MIXTURES

pH at 20°C.	M/10 KH phthalate in cc.*	M/10 NaOH in cc.*
4.0	50	0.4
4.2	50	3.7
4.4	50	7.5
4.6	50	12.0
4.7	50	15.0
4.8	50	17.7
4.9	50	20.8
5.0	50	23.9
5.1	50	26.8
5.2	50	30.0
5.3	50	32.6
5.4	50	35.5

	M/15 KH_2PO_4 in cc.**	M/15 Na_2HPO_4 in cc.
5.2	98.2	1.8
5.3	97.4	2.6
5.4	96.4	3.6
5.5	95.8	4.2
5.6	94.8	5.2
5.7	93.3	6.7
5.8	91.6	8.4
5.9	90.0	10.0

pH at 20°C.	M/15 KH_2PO_4 in cc.**	M/15 Na_2HPO_4 in cc.
6.0	87.1	12.9
6.1	84.0	16.0
6.2	80.9	19.1
6.3	77.4	22.6
6.4	73.0	27.0
6.5	68.2	31.8
6.6	63.0	37.0
6.7	56.6	43.4
6.8	50.8	49.2
6.9	44.8	55.2
7.0	38.9	61.1
7.1	33.4	66.6
7.2	28.0	72.0
7.3	23.2	76.8
7.4	19.2	80.8
7.5	15.9	84.1
7.6	13.0	87.0
7.7	10.6	89.4
7.8	8.5	91.5
7.9	6.9	93.1
8.0	5.6	94.4
8.1	4.3	95.7
8.2	3.2	96.8
8.3	2.5	97.5
8.4	2.0	98.0

*Make each mixture of KH phthalate and NaOH up to 100 cc. with distilled water.
**M/15 NaH_2PO_4 may be used.

6. After an object is weighed, first read the weights from the vacant spaces where they are kept and second read again as the weights are returned to their places.
7. Objects to be weighed must never be placed in direct contact with the pans unless they are metallic, glass, or porcelain.
8. Hot objects cannot be accurately weighed.
9. Never leave the beam resting on the knife-edge when not in use and never leave the weights on the pan.

B. *Determining the Zero Point.*

1. Release the beam, then the pans.
2. The door of the balance case must always be closed when observing the swinging of the pointer.
3. If the pointer does not oscillate when the beam and pans are released, raise the door of the case a few inches, fan the air over the right pan with one hand, and then close the door.
4. After a few excursions of the pointer, begin to note and record the number of scale divisions the pointer reaches in its swings past the center of the scale, estimating to the tenths of a division.
5. Place the readings to the right of the center zero point in a column headed R and those to the left in one headed L.
6. Take 3 or 4 readings on one side and a greater number by one on the other side.
7. Add the columns and divide each sum by the number of readings taken on that side to obtain the average.
8. Add the 2 averages and divide by 2, then subtract from the greater average obtained in (7).
9. The result is the distance from the center of the scale on the side of the longer swing at which the pointer would stop if the beam were to come to rest, that is, the zero point.
10. If this is more than half a division from the center, it should be brought nearer by adjusting the nut on the screw projecting from the end of the beam.

C. *Weighing.*

1. Watch glasses are usually used in weighing. Two are accurately balanced against each other.
2. Add the required weights to the right hand pan.
3. Remove the watch glass from the left pan with forceps and add an approximated amount of solid material with a spatula. Only the last trace should be added when the watch glass is on the balance.
4. After the exact amount has been added, as determined by the swings of the balance needle, the weights are replaced in their box and the rider returned to its zero mark.

ransfer the contents of the watch glass to e required volumetric flask with the aid a funnel with a long wide stem. The lid must be in the form of powder or nall crystals.

ash the watch glass and funnel thoroughly, llecting the washings quantitatively in the lumetric flask.

VII. Visual Colorimeter.

hysical Laws Applied to Colorimetry.

ambert's Law: the amount of light absorbed a substance is proportional to the thickness f the absorbing substance.

eer's Law: light is absorbed in direct proortion to the number of molecules through hich light passes. Thus, in passing through colored medium, light is absorbed in direct roportion to the concentration of the colored ıbstance.

rinciple: A combination of the two above ws may be applied to the colorimeter. When ne same intensity of color by transmitted ght is produced in two columns of solutions ontaining the same chromogen in different oncentrations, the concentrations are inersely proportional to the length of the espective columns. This mathematically may e stated as follows:

$$\text{onc. S: Conc. U :: RU: RS or } \frac{\text{Conc. S}}{\text{Conc. U}} = \frac{\text{RU}}{\text{RS}} \text{ or}$$

$$\text{onc. S} \times \text{RS} = \text{Conc. U} \times \text{RU.}$$

onc. S = concentration of standard.
onc. U = concentration of unknown.
U = Reading of unknown on colorimeter.
S = Reading of standard on colorimeter.

pplication of Lambert and Beer's Laws.

When the dilution of the unknown and of he standard is the same and the result is to e expressed in units of standard, use the ollowing formula:

$$\text{onc. U} = \frac{\text{RS}}{\text{RU}} \times \text{Conc. S.}$$

When the dilution of the unknown and the tandard is the same and the result is to be xpressed in units of standard per 100 cc. of lood, use the following formula:

$$\text{onc. U.} = \frac{\text{RS}}{\text{RU}} \times \text{Conc. S} \times \frac{100}{\text{V}}$$

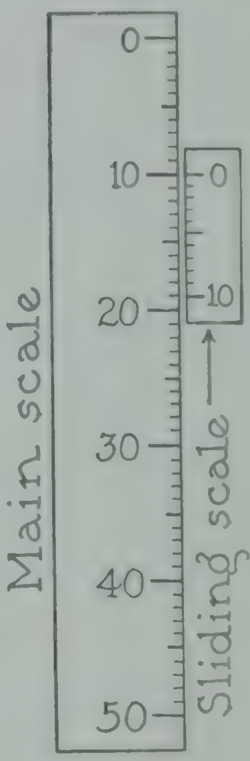
/ = volume of blood used in test.
When a protein-free filtrate is used, divide quanity of filtrate used by dilution to obtain volume of lood used; i.e., 5 cc. of filtrate is equivalent to 1/10 f 5 or 0.5 cc. of blood.)

When the dilution of the unknown and that of the standard differ and the result is to be expressed in units of standard per 100 cc. of lood, use the following formula:

$$\text{Conc. U} = \frac{\text{RS}}{\text{RU}} \times \text{Conc. S} \times \frac{100}{\text{V}} \times \frac{\text{Dil. U}}{\text{Dil. S.}}$$

D. ***Reading the Colorimeter Scale.***

1. The reading will consist of a whole number and a decimal.
2. Each division on the main scale is 1 mm. However, the small sliding scale (vernier) covers only 9 divisions on the main scale (see illustration); therefore, each division is 0.1 mm. less than one division on the main scale. If the zero line on the sliding scale is moved just beyond the 10 division on the main scale, it will be found that line 1 will be the division on the sliding scale that will coincide with a division on the main scale. To do this the plunger must have been moved 0.1 mm. and the reading is 10.1. If moved so that line 6 on the sliding scale coincides with a division on the main scale, the reading is 10.6, since the plunger was moved 0.6 mm. to bring line 6 on a level with a division on the main scale.



E. ***Filling Colorimeter Cups and Cleaning.***

1. Do not fill the cup so full that the solution reaches the top of the plunger when it is lowered into the solution. Do not fill beyond the beginning of the flared part of the cup.
2. To avoid spotting the instrument with chemicals, wipe the outside of the cup before placing it on the stage.
3. Do *not* allow the cup to overflow during the color matching.
4. Instantly remove any solution which falls on the light filter or mirror. Clean the whole mirror so that the surface will be uniform. If a lamp is used in place of a mirror, be sure it is cleaned promptly.
5. After using, the cups are washed with distilled water and the plungers rinsed at least 3 times with distilled water. Solutions must not be left in the cups any longer than necessary.
6. Wipe plungers and cups dry with a piece of soft cloth.
7. When not in use, keep the colorimeter in a dust proof place.

F. *Standardization.*

1. If daylight is used, a north light is best. Never place the colorimeter in the sunlight.
2. If artificial light is used, place colorimeter in the darkest place in the room so that daylight will not interfere.
3. When the cups are empty and in position so that the plungers touch the bottoms, the scale must read zero. If not, adjust the scales so they do read zero.
4. Place a small amount of the standard solution of the determination in each cup and rinse the plungers with it.
5. Discard this rinse solution and place more of the standard solution in each cup.
6. Place the cups in the colorimeter, set each at the height on the scale (20 mm.) to be used for the standard when reading the unknown, and adjust the light so the two cups are a perfect match.
    a. There must be no bubbles under the plunger.
    b. The cups and plungers must be scrupulously clean before filling.
    c. There should be no turbidity in the solution.

G. *Reading the Unknown.*

1. After adjusting the light with the standard solutions, empty the cup on the right side and rinse the cup and plunger with a small amount of the unknown solution. Discard and fill again for the reading.
2. When several determinations are made with the same standard, rinse the right hand cup and plunger each time with the unknown.
3. Do not look for more than a moment at a color. Take a number of rapid readings rather than a few unduly careful ones.
4. Approach the match from both the darker and lighter sides.
5. If the first reading does not approach the successive readings, disregard it in the average.
6. Average the readings.
7. Due to an error in matching colors of unequal column heights, it is important that the strengths of the unknown and standard be approximately the same so that the unknown reading is within ± 3 mm. (at the most ± 5) of the standard.

VIII. Photoelectric Colorimeter.

A. *Principle.*

1. Light creates an electric potential in a photoelectric cell. Since the current output of the cell depends upon the intensity of the light reaching it, the cell will be affected by colored solutions and also turbid solutions interposed between it and the light source. The light intensity is measured by means of an electrical circuit which includes a resistance coil and a galvanometer. The indicator of the galvanometer swings over a scale indicating the amount of current generated in arbitrary units (one cell type), or the indicator is controlled by a variable resistance and the amount of resistance is measured on an arbitrary scale (two cell type).
2. For monochromatic light, that is, one color provided by the use of filters, the ratio of the intensity of light leaving a colored solution to that entering it is a logarithmic function of its concentration. When appropriate light filters covering a narrow band of wave lengths are used, Beer's law for colorimetric procedures is valid for the photoelectric colorimeter.

B. *Types of Photoelectric Colorimeters.*

1. *Single Cell Type* (Example: Evelyn).
    a. It has one cell and a constant light source of low intensity and voltage (storage battery).
    b. The galvanometer has a unit scale and readings plotted against concentrations on semilogarithmic paper (1 cycle × 70 divisions) will form a straight line. Such a curve must be made by using various dilutions of standards for each procedure. Values for unknowns are interpolated from these graphs.
        1) It is possible to calculate unknowns without a curve by using one or more standards and the following formula:

$$\text{Conc. S} \times \frac{\text{L value of unknown}}{\text{L value of standard}} \times \frac{100}{\text{V}} = \text{mg. \%}$$

$\text{L} = 2 - \log$ of the galvanometer reading.
$\text{V} =$ Volume of blood used in test.

        2) This is only accurate when the concentration of the standard approximates that of the unknown.
2. *Two Cell Type* (Example: Klett-Summerson).
    a. Due to the 2 cells which produce a constant source of light, these photoelectric colorimeters can be used on alternating or direct current.
    b. The galvanometer has a logarithmic scale, such that readings plotted against concentrations on cross-section paper will form a straight line.
    c. The concentration of the unknown solutions is obtained by multiplying the galvanometer readings by the proper factor predetermined from a standard.

recautions in Using a Photoelectric Colorneter.

nstrument must be placed away from either echanical or electrical vibrations.

must not have a strong light overhead and irect sunlight must not be allowed to strike e colorimeter tube.

he filter used for each procedure is the one hich has a spectral transmission opposite to hat of the solution being measured, i.e., the lter which transmits the most light over the ange in which the solution absorbs the most ght.

filter must be placed in the machine before urning on the light to protect the photolectric cell.

nly test tubes made for the particular type f photoelectric colorimeter should be used nd these must be checked for uniformity of hysical dimensions. (Some instruments use uvettes which are checked at the factory.)

- They must be kept scrupulously clean, both inside and out.
- It is possible to add too much or too little fluid to the tubes or cuvettes for correct light transmission.
- After filling the tube or cuvette, the outer surface must be wiped clean of finger marks with a lint-free cloth and held up to the light to be sure that the solution is free of air bubbles.
- Before using new tubes, check for uniformity of physical dimensions.
    1) Place 15 cc. of water in a clean tube, and polish the lower end of the tube with a clean cloth.
    2) Using any filter, set the galvanometer at exactly 50, the utmost care must be used to keep this center setting.
    3) Insert the tubes one at a time, the galvanometer will move about 10 divisions.
    4) Rotate each one slowly through one full revolution to determine the position of greatest stability of reading, and make a diamond pencil mark at the upper end of the tube so that the tube can be placed in the same relative position at all times.
    5) Record the number of each tube and the reading obtained, discard all tubes which fluctuate more than 0.25 division from the mean, as well as any tube which fluctuates more than 0.5 division when rotated through 360 degrees.

is very important that each test be read nder the same conditions (length of time and temperature) that the standard curve was determined. These factors affect the color in some tests causing it to be progressively stronger or weaker.

7. Periodically standards should be set up with unknowns as a check on reagents and technique.

IX. Reporting Results of Chemical Determinations.

A. *Methods.*

1. *Per cent.*
    a. Most constituents of the blood are reported in milligrams or grams in 100 cc. of whole blood, plasma, or serum.
    b. Volume per cent is used for gas analyses.
2. *Units* are used in most enzyme determinations; the unit is defined for each test.
3. *Milliequivalents.*
    a. A milliequivalent is one one-thousandth of a gram-equivalent weight.
    b. Milliequivalents per liter are calculated by dividing the milligrams per liter by the equivalent weight of the element.
    c. The equivalent weight of an element is its atomic or formula weight divided by its valence. (The valence of an element or radical is that property which is measured by the number of replaceable hydrogen atoms or the equivalent.)
4. *Millimol.*
    a. A millimol is one one-thousandth part of a gram-molecular weight.
    b. Millimols per liter are calculated by dividing the milligrams per liter by the atomic weight of the element.

B. The following list gives the corresponding values for a few constituents of blood.

	Mg. per 100 cc. of serum	Milliequivalent per liter of serum	Millimols per liter of serum
Chloride	580-630 (NaCl)	99-108	99-108
Calcium	9.5-11	4.7-5.5	2.35-2.75
Sodium	325-350	141-152	141-152
Potassium	18-21	4.6-5.6	4.6-5.6
Carbon dioxide content	56-65 vol.%	25-29	25-29

Protein-Free Filtrate

I. Principle: The total proteins of whole blood, serum, or other body fluids are removed by precipitation with tungstic acid (formed by the interaction of sodium tungstate and sulfuric acid) and filtration.

$Na_2WO_4 + H_2SO_4 \rightarrow H_2WO_4 + Na_2SO_4$.

II. Whole Blood.

A. *General Considerations.*

1. Make filtrate within 2 hours after withdrawal of blood from the patient, except filtrates for sugar determinations which must be made within 30 minutes.
2. Filtrates may be kept overnight in the refrigerator if a few drops of toluene are added as a preservative.

B. *Haden's Modification of Folin-Wu Method.*

1. Shake the bottle containing the oxalated blood until the blood is well mixed.
2. Measure 1 volume of oxalated blood with an Ostwald pipette into an Erlenmeyer flask which will hold at least 20 times the volume of blood used. Allow the blood to flow slowly from the pipette so that a minimum amount of blood will cling to the inside of the pipette.
3. With continual shaking of the flask, slowly add 8 volumes of N/12 sulfuric acid from a burette. Continue to shake for 2 minutes or until complete hemolysis has taken place.
4. Add 1 volume of 10% sodium tungstate solution slowly while shaking the flask.
5. This makes a 1-10 dilution; in every 10 cc. of filtrate there is the equivalent of 1 cc. of blood.
6. Insert a rubber stopper and shake well.
    a. The metallic click and complete absence of foam on the surface of the mixture are indicative of complete precipitation of protein.
    b. Since precipitation of protein is complete only at the isoelectric point, it is very important that the acid and base (Na_2WO_4) be *accurately* made and pipetted.
    c. The mixture should show no acid when tested with Congo red paper.
7. Let stand 10 to 20 minutes (20 minutes if uric acid is to be determined).
8. Shake the mixture again and filter through Whatman No. 1 filter paper.
    a. The filter paper should be large enough so that all the mixture can be filtered at once thus obtaining the filtrate more quickly and in larger amount.
    b. Pour the mixture slowly onto the triple portion of the filter paper in order that the paper will be wet above the level of the mixture.
    c. Allow the first drops to filter back into the original container before collecting the filtrate.
    d. Cover the funnel with a watch gla
    prevent evaporation.
    e. When there is a very small amount
    mixture, centrifugation may be used.
9. If the above procedure is properly car
out, the resultant filtrate is clear and
not need to be refiltered.
    a. A brownish filtrate usually indicates
    of three things: insufficient acid
    used, there was too much oxalate in
    original container for the amount
    blood obtained, or impure tungstate
    used.
    b. If *necessary*, pour filtrate back into m
    ture and add 10% sulfuric acid drop
    drop until a slight tinge of blue is o
    tained on Congo red paper; then refilt
10. Approximately 4 cc. of filtrate are obtain
when 1 cc. of blood is used.

C. *Folin-Wu Method.*

1. Follow directions 1 and 2 under Haden
modification.
2. With continual shaking of the flask, slow
add 7 volumes of water.
3. Continue to shake for 2 minutes or un
complete hemolysis has taken place.
4. Add 1 volume of 10% sodium tungstate s
lution and mix.
5. With continual shaking of the flask, a
slowly 1 volume of 2/3 N sulfuric aci
6. Continue as directed in Haden's modificati
beginning with 6.

D. *Solutions.*

1. *Sulfuric Acid N/12 (0.0833 N).*
    a. Add 2.5 cc. of conc. H_2SO_4 to abo
    700 cc. of water in a liter volumetr
    flask, cool, and dilute to volume.
    b. After thorough shaking and standi
    overnight, titrate with 0.1 N NaOH u
    ing phenolphthalein for the indicator (
    cc. of N/12 H_2SO_4 requires 16.67 c
    of 0.1 N NaOH for neutralization).
    c. Adjust acid to N/12.
2. *Sulfuric Acid 2/3 N (0.666 N).*
    a. Add 20 cc. of conc. H_2SO_4 to about 7
    cc. of water in a liter volumetric flas
    cool, and dilute to volume.
    b. After thorough shaking and standi
    overnight, titrate with N NaOH usi
    phenolphthalein for the indicator (20 c
    of 2/3 N H_2SO_4 requires 13.33 cc.
    N NaOH for neutralization).
    c. Adjust acid to 2/3 N.
3. *Sodium Tungstate Solution—10%.*
    a. Make a 20% solution by dissolving 2
    gm. of sodium tungstate (Na_2WO
    $2H_2O$, special according to Folin) in abo

700 cc. of water in a 1 liter volumetric flask and dilute to volume. Allow to stand several days before using so that any precipitate may settle to the bottom of the flask.

Make a 10% solution by diluting 1 volume of the clear supernatant fluid of the 20% solution with 1 volume of water.

A satisfactory sodium tungstate will give a solution which is neutral or faintly alkaline to phenolphthalein.

1) If the solution is acid, titrate with 0.1 N NaOH and add a proportional amount of N NaOH to the main solution to make neutral.
2) If the solution is alkaline, not more than 0.4 cc. of 0.1 N HCl should be required to neutralize 10 cc. of the solution. If more is required, the solution should be discarded.

III. Plasma or Serum.

Method.

Centrifuge blood to separate plasma or serum.
Measure 2 volumes of plasma or serum with an Ostwald pipette into an Erlenmeyer flask which is large enough to contain at least 20 times the volume of plasma or serum used.
Add 9 volumes of distilled water and mix.
Add 8 volumes of N/12 sulfuric acid slowly from a burette and mix.
Add 1 volume of 10% sodium tungstate solution slowly while shaking the flask.
Insert a rubber stopper and shake well.
This makes a 1-10 dilution.
Filter as described under whole blood filtrate using Whatman No. 40 filter paper.

Solutions—see solutions under whole blood.

Carbohydrates

Blood Sugar (Original Folin-Wu Method).

Principle: A portion of the protein-free blood filtrate is heated with an alkaline cupric solution. The cuprous oxide produced by the reaction of cupric hydroxide and glucose reduces phosphomolybdic acid to phosphomolybdous acid which is blue. This blue color is compared with that of a standard solution of glucose.

General Considerations.

The filtrate from blood specimens for sugar determinations must be made up at once unless the sodium fluoride and thymol mixture is used as an anticoagulant.

2. Blood filtrates containing a few drops of toluene may be kept in the refrigerator for 24 hours before analyzing without any appreciable change. Bring to room temperature before pipetting.
3. All that is reported as blood sugar is not glucose, but the sum total of reducing substances encountered during the determination.
   a. The nonfermentable portion of the reducing substances is composed of glutathione, ergothioneine, creatinine, and unknown substances.
   b. The concentration of the nonfermentable portion varies from 10 to 30 mg. per cent.
4. Blood from the finger or ear (capillary or arterial blood) gives higher values than venous blood except in the fasting state when they are the same. (See Interpretation of Glucose Tolerance, page 269.)
5. *Sources of Error.*
   a. Directions for heating and cooling the solution must be followed with scrupulous care because variations in temperature or time affect the results significantly.
   b. Cuprous compounds produced by sugar in alkaline copper solutions are readily oxidized to the cupric state when exposed to air; therefore, shaking is avoided after the tube is placed in the water bath.

C. *Macromethod.*

1. Place 2 cc. of protein-free filtrate in a Folin-Wu blood sugar tube graduated at 12.5 and 25 cc.
2. Place 2 cc. of the 3 standard glucose solutions (0.2 mg., 0.3 mg., and 0.4 mg.) respectively in each of 3 test tubes graduated as above.
3. Add 2 cc. of alkaline copper tartrate solution to each tube and mix well by gently shaking the tubes. (The surface of the mixture must reach the constricted part of the tube.)
4. Heat immediately in a boiling water bath for 6 minutes.
5. Cool, without shaking, in a cold water bath for 3 minutes.
6. To each tube add 2 cc. of phosphomolybdic acid reagent.
7. When vigorous effervescence has ceased, dilute to the 25 cc. mark with water.
8. Insert a rubber stopper and mix each tube thoroughly by repeated inversion.
9. Within 10 minutes compare the unknown in the colorimeter with the standard that most closely approximates its color.
10. Set standard at 20 mm.

11. *Calculation:*
    a. $\frac{RS}{RU} \times 0.2 \times \frac{100}{0.2} = \frac{2000}{RU} = \text{mg.\%}.$
    b. $\frac{RS}{RU} \times 0.3 \times \frac{100}{0.2} = \frac{3000}{RU} = \text{mg.\%}.$
    c. $\frac{RS}{RU} \times 0.4 \times \frac{100}{0.2} = \frac{4000}{RU} = \text{mg.\%}.$
    d. These results are in error when the standard and the unknown do not read close together.

D. *Micromethod.*

1. Pipette 0.8 cc. of $N/12\ H_2SO_4$ into a conical tipped centrifuge tube.
2. Add 0.1 cc. of whole blood using a special microsugar pipette. Place tip of the pipette to the bottom of the tube and let blood flow out to prevent as much as possible mixing with the acid. Rinse the blood from the pipette twice with the clear acid above the blood.
3. Mix thoroughly with a stirring rod and allow to stand until completely hemolyzed without removing the stirring rod.
4. Add 0.1 cc. of 10% sodium tungstate solution allowing it to run down the side of the stirring rod.
5. Mix thoroughly with the stirring rod and remove the rod.
6. Centrifuge until the supernatant fluid is clear.
7. Pipette 0.5 cc. of this blood filtrate into a straight pyrex test tube graduated at 4, 8, and 16 cc. (The regular Folin-Wu sugar tubes cannot be used because a dilution to 12.5 cc. is too great.)
8. Pipette 1 cc. of the standard glucose solutions (0.1 mg., 0.15 mg., and 0.2 mg.) respectively into each of 3 similar tubes.
9. Proceed exactly as described in the macromethod except add 0.5 cc. of alkaline copper tartrate solution and 0.5 cc. of phosphomolybdic acid to the tubes containing the blood filtrates (not the standards) and make the final dilution to 8 cc. with water.
10. Use 1 cc. of all solutions in the tubes containing the standards and dilute to 16 cc.
11. This method can be used as a macromethod by using 1 cc. of blood filtrate and 1 cc. of the reagents and diluting to 16 cc. However, in the calculation 0.1 cc. must be substituted for 0.05 cc. and 1 for 8/16 in the formula.
12. *Calculation:*
    a. $\frac{RS}{RU} \times 0.1 \times \frac{100}{0.05} \times \frac{8}{16} = \frac{2000}{RU} = \text{mg.\%}.$
    b. $\frac{RS}{RU} \times 0.15 \times \frac{100}{0.05} \times \frac{8}{16} = \frac{3000}{RU} = \text{mg.\%}.$
    c. $\frac{RS}{RU} \times 0.2 \times \frac{100}{0.05} \times \frac{8}{16} = \frac{4000}{RU} = \text{mg.\%}.$

E. *Solutions.*

1. *Alkaline Copper Tartrate Solution.*
    a. Place 40 gm. of pure anhydrous sodi carbonate in a beaker and add 400 cc. water. Dissolve, use heat if necessar
    b. Add 7.5 gm. of tartaric acid $(C_4H_6O$ when completely dissolved, transfer to liter volumetric flask.
    c. Add slowly with constant stirring 4. gm. of crystalline copper sulfate $(CuSO_4 \cdot 5H_2O)$ which has been previously dissolved in about 200 cc. of water with heat and then cooled.
    d. Mix and dilute to 1 liter with water.
2. *Phosphomolybdic Acid Solution.*
    a. Place 70 gm. of molybdic acid (H_2MoO_4) and 10 gm. of sodium tungstate (Na_2WO_4) in a 2 liter beaker.
    b. Add 400 cc. of 10% NaOH and 400 cc. of water.
    c. Boil vigorously for 40 to 60 minutes to remove all the ammonia present in the molybdic acid.
    d. Cover and place in the refrigerator overnight.
    e. Filter (to remove light precipitate of iron and silicate) into a liter volumetric flask, wash the filter paper with water, and dilute the filtrate to about 700 cc.
    f. Add 250 cc. of 85% phosphoric acid.
    g. Mix, cool to 20°C., and dilute to 1 liter with water.
    h. This solution should not be blue.
3. *Standard Glucose Solutions.*
    a. *Stock glucose solution—1%.*
        1) Weigh exactly 1 gm. of anhydrous glucose (c.p.) which has been dried in a desiccator for 3 days and place in a 100 cc. volumetric flask.
        2) Make up to volume at 20°C. with 0.25% benzoic acid solution. (Dissolve 2.5 gm. of benzoic acid in 1 liter of boiling water.)
        3) Keeps indefinitely.
    b. *Three working standard glucose solutions.*
        1) *Weak standard*—place 10 cc. of the stock glucose solution in a liter volumetric flask and dilute to volume with 0.25% benzoic acid (1 cc. = 0.1 mg.)
        2) *Intermediate standard*—place 15 cc. of the stock glucose solution in a liter volumetric flask and dilute to volume with 0.25% benzoic acid (1 cc. = 0.1 mg.).
        3) *Strong standard*—place 20 cc. of the stock glucose solution in a liter volumetric flask and dilute to volume with 0.25% benzoic acid (1 cc. = 0.2 mg.).

. Blood Sugar (Modified Folin-Wu Method).

rinciple and General Considerations are e same as under original Folin-Wu method.

acromethod.

lace 1 cc. of protein-free filtrate in a pyex test tube graduated at 4, 8, and 16 cc.

lace 1 cc. of the 3 standard glucose solutions 0.1 mg., 0.15 mg., and 0.2 mg.) respectively n each of 3 test tubes (graduated as above).

Add 1 cc. of freshly prepared copper tarrate solution to each tube and mix well by apping the tubes.

Heat immediately in a boiling water bath for 8 minutes.

Cool, without shaking, in a cold water bath for 3 minutes.

Add to each tube 2 cc. of conc. acid molybdate solution and allow to stand for 1 minute.

Dilute to the 16 cc. mark with the dilute acid molybdate solution.

Mix thoroughly by inverting 3 times and allow to stand 12 minutes.

Read in the colorimeter with the standard that most closely approximates its color.

Set standard at 20 mm.

Calculation:

a. $\frac{RS}{RU} \times 0.1 \times \frac{100}{0.1} = \frac{2000}{RU} = \text{mg.\%.}$

b. $\frac{RS}{RU} \times 0.15 \times \frac{100}{0.1} = \frac{3000}{RU} = \text{mg.\%.}$

c. $\frac{RS}{RU} \times 0.2 \times \frac{100}{0.1} = \frac{4000}{RU} = \text{mg.\%.}$

Micromethod.

Make filtrate as described under micromethod for sugar (original Folin-Wu method).

Using 0.5 cc. of blood filtrate and 1 cc. of standard glucose solutions, proceed exactly as in the macromethod (modified Folin-Wu method). Be careful to use 0.5 cc. of freshly prepared copper tartrate solution and 1 cc. of conc. acid molybdate solution for the tubes containing blood filtrate and dilute to 8 cc. with dilute acid molybdate solution. Use same proportions for standards as in macromethod and dilute to 16 cc.

Calculation is the same as for the original Folin-Wu micromethod.

Solutions for Modified Method.

Alkaline Copper Tartrate Solution.

a. *Copper sulfate—5%* (Solution A).
   1) Dissolve 50 gm. of crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$) in water in a liter volumetric flask.
   2) Add 1 drop of conc. sulfuric acid and dilute to volume with water.

b. *Alkaline tartrate* (Solution B).
   1) Transfer 35 gm. of anhydrous sodium carbonate to a liter volumetric flask.
   2) Add about 200 cc. of water until the carbonate is dissolved.
   3) Add 13 gm. of sodium tartrate, 11 gm. of sodium bicarbonate and about 700 cc. of water.
   4) Dissolve and dilute to volume with water.

c. *Alkaline copper tartrate solution*—Make fresh on day of analysis.
   1) Place 12.5 cc. of solution B in a 25 cc. volumetric flask.
   2) Add 2.5 cc. of solution A, mix, and dilute to mark with solution B.

2. *Purified Acid Molybdate Reagent.*
   a. *Stock brominated sodium molybdate solution.*
      1) Dissolve 300 gm. of sodium molybdate in water in a liter volumetric flask.
      2) Add 2 or 3 drops of liquid bromine (be careful).
      3) Dilute to mark with water and mix.
      4) A precipitate may settle out on long standing.
   b. *Concentrated acid molybdate solution.*
      1) To 500 cc. of clear supernatant brominated sodium molybdate solution in a liter volumetric flask add 225 cc. of 85% phosphoric acid.
      2) The solution becomes yellow because of liberated bromine.
      3) Add 150 cc. of cooled 25% sulfuric acid.
      4) Aerate about 4 to 5 hours to remove the bromine, then add 75 cc. of glacial acetic acid, mix, and dilute to volume with water.
   c. *Dilute acid molybdate solution.*
      1) Place 200 cc. of concentrated acid molybdate solution in a liter volumetric flask.
      2) Dilute to volume with water.

3. *Standard solutions*—same as those in original Folin-Wu method.

III. Blood Sugar (Photoelectric Colorimeter Method).

A. ***Principle and General Considerations*** are the same as under original Folin-Wu method.

B. ***Method.***

1. Place 1 cc. of protein-free filtrate in a Folin-Wu blood sugar tube graduated at 12.5 and 25 cc. Care should be taken to pipette the solution low in the tube and to get as little as possible on the sides of the tube.

2. Place 1 cc. of 2 standard glucose solutions (0.1 mg. and 0.2 mg.) respectively in each of 2 tubes graduated as above.
3. Prepare a blank by placing 1 cc. of water in a similar tube.
4. Add slowly 1 cc. of freshly prepared alkaline copper tartrate solution to each tube, allowing solution to run down the side of the tube and rotating the tube to wash down all the filtrate or standard solution already added.
5. Mix by shaking gently.
6. Immediately place tubes in boiling water for 8 minutes.
7. Cool, without shaking, in a cold water bath for 3 minutes.
8. Add to each tube 2 cc. of conc. acid molybdate solution and shake until effervescence ceases.
9. Place in boiling water for 5 minutes; this stabilizes the blue color.
10. Place in a cold (10°C.) water bath for 2 minutes.
11. Dilute to the 25 cc. mark with water which has been kept in the refrigerator.
12. Mix thoroughly by repeated inversion.
13. Keep all tubes in the cold water bath, removing each just before reading in the photoelectric colorimeter.
14. Use filter No. 420.
15. Place some of the "blank" in a colorimeter tube and adjust the galvanometer to 100.
16. Each standard is read and the results checked on the table of values. If the standards vary appreciably from their correct readings, the determinations must be repeated.
17. Read unknowns and obtain mg. per cent of glucose from the table of values.

C. *Calibration of Standard Curve.*

1. *Stock Standard Glucose Solution*—0.5%.
   a. Weigh exactly 1 gm. of anhydrous glucose (c.p.) which has been dried in a desiccator for 3 days.
   b. Place in a 200 cc. volumetric flask and make to volume with 0.25% benzoic acid at 20°C.
2. Prepare a series of dilute standard glucose solutions as follows:

cc. of 0.5% glucose standard solution	Final volume with 0.25% benzoic acid	mg. glucose per cc. of dilute standard solution
2	500	0.02
2	250	0.04
3	250	0.06
4	250	0.08
5	250	0.10
3	100	0.15
10	250	0.20
6	100	0.30

3. Using 1 cc. portions of each of these dilute standard solutions, determine the galvanometer readings by the above method.
4. Repeat several times with each concentration.
5. Prepare several new stock standard glucose solutions, make new dilute standard solutions from each, and repeat tests several times on each dilute standard solution.
6. Average the galvanometer readings for each dilute standard solution and plot on semi-logarithmic paper drawing a straight line through the points.
7. Prepare a table of values for the mg. per cent of glucose for each division on the galvanometer. The mg. per cc. of the dilute standard solutions is multiplied by 100 to obtain mg. per cent of glucose.

D. *Solutions*—same as for Modified Folin-Wu method.

IV. Interpretation of Blood Sugar Findings.

A. *Normal Values:*

1. *Original Folin-Wu:* 80—120 mg. per cent.
2. *Modified Folin-Wu:* 70—110 mg. per cent.
3. *Microsugars* after a meal are higher than venous blood sugars because the blood taken from the finger is a mixture of venous and arterial blood. The microsugar is the same as the venous blood sugar in the fasting state.

B. *Increase Values in:*

Diabetes mellitus
Hyperpituitarism
Increased intracranial pressure
Coronary thrombosis
Hyperthyroidism
Chronic nephritis
Urinary obstruction
Shock
Infections
First 24 hours after severe burns

C. *Decreased Values in:*

Insulin effect
Hyperinsulinism
Hepatic insufficiency
Pancreatic adenoma
Carcinoma of islet tissue
Addison's disease
Hypothyroidism
Starvation
Hypopituitarism
Pernicious vomiting of pregnancy

D. *Renal Threshold.*

1. Normal renal threshold for blood glucose is 160-200 mg. per 100 cc.
2. It is usually raised in diabetes mellitus and late nephritis so urine sugar can not be entirely relied upon as a measure of blood sugar.

enal diabetes or renal glucosuria is a condi- on in which the threshold is lowered and gar appears in the urine although it is not creased in the blood.

V. Spinal Fluid Sugar.

rinciple: Sugar is determined on a protein- ee filtrate of spinal fluid in the same man- er as blood sugar is determined.

eneral Considerations.

he sugar determination must be made with- 30 minutes after the withdrawal of the inal fluid.

blood sugar determination should also be ade on a sample of blood withdrawn at e same time the spinal fluid is obtained. ormally the glucose in spinal fluid is 60 per ent of that in the blood.

ethod.

lake a protein-free filtrate (1-5 dilution).

. Place 1 cc. of spinal fluid in a test tube, add 3.5 cc. of water, and then add 0.25 cc. of 2/3 N sulfuric acid while shaking the tube.

. Add 0.25 cc. of 10% sodium tungstate solution dropwise, stopper, and shake.

. Allow to stand 5 minutes and filter.

he supernatant fluid, after the precipitation nd centrifugation of total protein in the pinal fluid, may be used for the sugar de- ermination.

etermine the sugar in the above filtrate or upernatant fluid in the same manner as that n a blood filtrate.

alculation:

a. When a 1-5 filtrate and 0.1 mg. standard glucose solution are used:

$$\frac{RS}{RU} \times 0.1 \times \frac{100}{0.2} = \frac{1000}{RU} = \text{mg.\%}.$$

b. When the supernatant fluid from the total protein determination and 0.1 mg. standard glucose solution are used:

$$\frac{RS}{RU} \times 0.1 \times \frac{100}{0.357} = \frac{560}{RU} = \text{mg.\%}.$$

c. When the photoelectric colorimeter method is used:
  1) For the 1-5 filtrate, multiply the reading from the glucose curve by 0.5.
  2) For the supernatant fluid from the total protein determination multiply the reading from the glucose curve by 0.28.

Interpretation—see Table 26 on page 154.

VI. Glucose Tolerance (Janney-Isaacson Single Oral Dose).

Principle: The patient is given a single dose of glucose by mouth after a specimen of fasting blood and urine have been obtained. Blood and urine samples are obtained 1/2, 1, 2, and 3 hours later for sugar determinations to detect hyperglycemia unresponsiveness which may be due to either insulin lack or insulin resistance.

B. *General Considerations.*

1. It is important that the individual be on a full, well-balanced diet (80 gm. protein and 300 gm. carbohydrates) for 3 days prior to the test.
2. Patient should have no food after the evening meal the night before the test.
3. Test should be done in the morning before the patient has exercised.
4. The patient should not exercise or smoke during the test.

C. *Method.*

1. Dissolve the correct amount of glucose or cerulose in water (2.5 cc. of water for each gm. of glucose) by heat and cool. Add the juice of 1 lemon.
   a. Individuals weighing less than 100 lbs. should receive 1.75 gm. of glucose per kilogram of body weight.
   b. Individuals weighing from 100 to 175 lbs. should receive 100 gm. of glucose.
   c. Individuals weighing over 175 lbs. should receive 120 gm. of glucose.
2. Blood (venous or capillary) is obtained before the glucose is given, and 30 minutes, 1, 2, and 3 hours after the glucose is taken.
3. The mg. of glucose per 100 cc. of blood is determined for each specimen.
4. A urine specimen is obtained at the same time each blood sample is drawn and is tested for sugar.
5. The patient may have a glass of water to drink 1 hour after the glucose is taken.
6. If pathologic hypoglycemia is suspected, obtain blood specimens also at 4 and 5 hours after the glucose is taken.

D. *Interpretation (see Fig. 27).*

1. Normally the venous blood sugar reaches the peak (120 to 160 mg.) between 30 minutes and 1 hour and returns to or near the fasting level at 2 hours.
2. The results of the micromethod using capillary blood (arterial) must be interpreted somewhat differently from those obtained when venous blood is used.
   a. Fasting values of capillary and venous blood are the same.
   b. The peak is reached about the same time regardless of the type of blood used.

   c. The peak with the capillary blood is usually 30 to 70 mg. higher than with venous blood.
   d. The capillary blood returns to normal 30 to 60 minutes later than venous blood.
3. Increased glucose tolerance (flat or inverted curve) is noted in hypothyroidism, hyperinsulinism, Addison's disease, hypopituitarism, sprue, idiopathic steatorrhea, celiac disease, muscular dystrophy, and anorexia nervosa (normal curve in intravenous method).
4. Decreased glucose tolerance (high or prolonged curve) is noted in diabetes mellitus, hyperthyroidism, hypercorticoadrenalism, hyperpituitarism, prolonged fasting, pregnancy, fatigue, emotional disturbance, shock, increased cerebral pressure, furunculosis, arsenic poisoning, carcinoma of the G.I. tract, chronic nephritis, and liver disease.

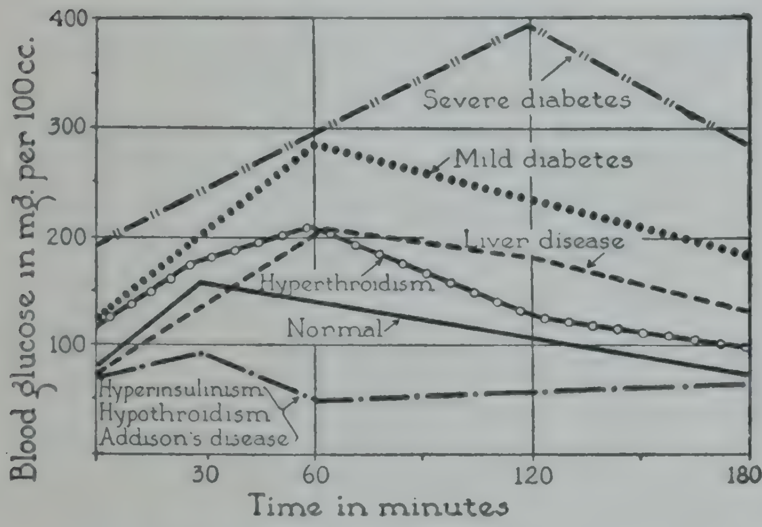


FIG. 27. TYPICAL GLUCOSE TOLERANCE CURVES.

VII. Glucose Tolerance (Exton-Rose Divided Oral Dose).

A. ***Principle:*** There are two opposing theories explaining the principle of this test:

1. Allen's paradoxical law of glucose: in normal individuals the more sugar given the more is utilized, while in diabetes the more given the less utilized.
2. Soskin's "over-production theory": in diabetes more sugar is utilized; therefore, the liver does not store sugar as glycogen but maintains a high blood sugar level.

B. ***General Considerations***—Same as in single oral dose test.

C. ***Method.***

1. Dissolve 100 gm. of glucose or cerulose in 650 cc. of water and add the juice of 1 lemon.
2. Obtain a fasting venous blood and urine spe men.
3. Administer orally one half of the glucose lution.
4. In 30 minutes collect venous blood and ur samples.
5. Give the remaining of the glucose solu and collect venous blood and urine samples minutes later.
6. Determine the sugar for each sample blood and test the urines for sugar.

D. ***Interpretation.***

1. Normal response to test.
   a. The fasting blood sugar must be withi the normal limits.
   b. The 30 minute blood sample should no exceed the fasting blood sugar more tha 75 mg.
   c. The 60 minute blood sugar should no exceed the 30 minute specimen more tha 10 mg.
   d. All the urine specimens should be nega tive for sugar.
2. Diabetes is present if the 60 minute bloo sugar exceeds the 30 minute sample 10 mg or more.
3. In renal glucosuria the blood sugars follo the normal curve, but the urine specimen are positive for sugar.
4. In alimentary glucosuria the blood sugar follow the normal curve even though th level is higher than normal, the fasting urin specimen is negative for sugar, while th hour urine specimen is positive for suga

VIII. Intravenous Glucose Tolerance Test.

A. ***Principle:*** The patient is given an intravenou injection of a 50% glucose solution after specimen of fasting blood is obtained. Th amount of glucose is determined in succes sive blood specimens.

B. ***General Considerations.***

1. Eliminates errors arising from differences i absorption in the oral methods.
2. Patient should be on a diet containing least 300 gm. of carbohydrates for 3 day previous to the test.
3. Patient should have no food after evenin meal the night before the test.
4. Care should be taken not to allow any the glucose solution to leak out of the vei into the tissue.

C. ***Microinterval Method*** **(McKean, Myer and Von der Heide).**

1. Calculate the cc. of a 50% glucose solutio necessary to inject 0.2 gm. of glucose p kilogram of body weight.

tain a fasting venous blood specimen.
ect the calculated amount of sterile glu-
se solution into an arm vein at a uniform
e in exactly 90 seconds.
ithin the next 3 minutes a vein in the
posite arm is punctured, and the needle
t in place while 2 to 3 cc. of blood is
llected at 3, 4, 5, 10, and 15 minutes,
ning from the end of the glucose injec-
n. (A 3-way metal stopcock attached to
syringe is convenient.)
etermine the mg. per cent of glucose in
ch specimen of blood.

***acrointerval Method* (Lozner, Winkler,
aylor, and Peters).**

otain a fasting venous blood specimen.
ject 50 cc. of a sterile 50% glucose solu-
on (25 gm.) intravenously during a period
2 minutes.
btain 3 to 4 cc. of venous blood from the
pposite arm at 15 and 30 minutes, 1, 2, and
hours after the injection of glucose.
etermine the mg. per cent of glucose in
ch specimen of blood.

terpretation.

the microinterval method the normal apex
f the curve is 175 mg. or lower, which is
eached in 3-5 minutes after the end of the
jection, and the curve returns to 125 mg.
r lower at 15 minutes.
the macrointerval method the normal
eak of the curve at 15 minutes ranges from
50 to 250 mg. per cent and returns to at
east 120 mg. per cent in 2 hours.
The 2 hour specimen is considered diagnostic in most cases.
If the 2 hour blood sugar is greater than 120 mg. per cent, the patient probably has diabetes mellitus, if less than 100 mg. per cent he probably does not, while if it falls between 100 and 120 mg. per cent the test is indeterminate.
he diabetic curve in both methods is simi-
ar to that obtained with the oral method.
f pathologic hypoglycemia is suspected, ob-
ain blood specimens hourly for at least 4
ours following the injection in the micro-
nterval method and obtain blood specimens
t 4 and 5 hours after the injection in the
macrointerval method.
Glycosuria occurs during the first hour in
normal persons.
Curves similar to those in diabetes may be
found in cardiac decompensation, hyper-
tension, cholecystitis, peptic ulcer, carcino-
ma, chronic encephalitis, and severe liver
disease.

IX. Insulin Tolerance.

A. ***Principle:*** The patient is injected subcutaneously or intravenously with insulin after a specimen of fasting blood has been obtained. Sugar determinations are made on successive blood specimens to measure the sensitivity of the blood glucose level to insulin and its responsiveness to an insulin induced hypoglycemia.

B. ***General Considerations.***

1. Patient should be on a diet containing at least 300 gm. of carbohydrates for 3 days previous to the test.
2. Patient should have no food after the evening meal of the night before the test.
3. The patient should be watched very closely (especially in the intravenous method) for hypoglycemic symptoms which are faintness, headache, dizziness, sensations of warmth, perspiration, palpitation, tremor, and visual disturbances. Clouding of the consciousness is a grave sign.
4. A 20 cc. ampule of a 50% sterile solution of glucose should be on hand to inject intravenously if the patient should show hypoglycemic symptoms.

C. ***Subcutaneous Method.***

1. Obtain a specimen of fasting blood.
2. Inject 10 units of insulin subcutaneously.
3. Take blood specimens at 1/2, 1, 2, and 3 hours following the injection of insulin.
4. Determine the amount of glucose in each specimen of blood by one of the methods for blood sugar.

D. ***Intravenous Method* (Fraser, Albright, and Smith).**

1. Obtain a specimen of fasting blood.
2. Inject intravenously 0.1 unit of insulin per kilogram of body weight. In cases of panhypopituitarism, Addison's disease, and hyperinsulinism, inject 0.01 unit per kilogram of body weight.
3. Take blood specimens at 20, 30, 45, 60, 90, and 120 minutes following the injection of insulin.
4. Determine the amount of glucose in each specimen of blood.

E. ***Interpretation.***

1. *Normal Response.*
    a. The glucose level falls to about 50 per cent of the fasting level in the 20 or 30 minute sample in both methods.
    b. The glucose level should be back to fasting level in 90 to 120 minutes after the insulin injection.

2. *Abnormal Response.*
   a. In insulin resistance the fall in glucose never reaches 50 per cent of the fasting level; this type of curve occurs in hyperpituitarism, hypercorticoadrenalism, and hyperthyroidism.
   b. In hypoglycemic unresponsiveness the glucose level falls below 50 per cent of the fasting level and fails to rise during the test or has a delayed rise; this type of curve occurs in hyperinsulinism, panhypopituitarism, and hypocorticoadrenalism (Addison's disease).

Nitrogenous Constituents

I. Nonprotein Nitrogen (N P N).

A. ***Principle:*** Nitrogen is determined in a portion of the blood filtrate by a micro-Kjeldahl method using an acid solution for the digestion which liberates ammonia; this in turn is converted to ammonium sulfate. The ammonia in the sulfate is converted into ammonium hydroxide by the base in the Nessler's solution.

$2\ NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4.$
$(NH_4)_2SO_4 + 2\ NaOH \rightarrow Na_2SO_4 + 2\ NH_4OH.$

The ammonium hydroxide reacts with the double iodide in the Nessler's solution to form dimercuric ammonium iodide. The solution of dimercuric ammonium iodide is colloidal and is matched against a known standard solution of ammonium sulfate which has been treated in the same manner. The colloidal dimercuric ammonium iodide solution must be kept cold to prevent precipitation.

$2\ NH_4OH \rightarrow 2\ NH_3 + 2\ H_2O.$
$2\ (HgI_2 \cdot 2KI) + 2\ NH_3 \rightarrow 2\ (NH_3 \cdot HgI_2) + 4\ KI.$
$2\ (NH_3 \cdot HgI_2) \rightarrow NH_2Hg_2I_3 + NH_4I.$
$2\ (HgI_2 \cdot 2KI) + 2\ NH_3 \rightarrow NH_2Hg_2I_3 + 4\ KI + NH_4I.$

B. ***General Considerations.***

1. Nonprotein nitrogen includes urea, uric acid, creatine, creatinine, ammonia, amino acids, and other nitrogenous substance ("rest N").
2. The "rest N" is of undetermined origin and constitutes almost half of the total nonprotein nitrogen of whole blood. This is usually increased in toxemias.
3. The ratio of urea nitrogen to nonprotein nitrogen may be used as a test for renal function. See renal function tests in Section on Urinalysis (p. 28).
4. *Sources of error.*
   a. If the tubes are not heated slowly, the liquid will boil above the 35 cc. mark and give low results.
   b. Excess of tungstic or sulfuric acid in filtrate will cause a yellow precip during digestion.
   c. Distilled water used in the test must free of any nitrogen compounds.
   d. Adding superoxyl immediately after moving flame from the tube.
   e. Contents of tubes must be cold (11°C before Nesslerizing or a cloudiness m develop.
   f. The level of the solution must be exact at the 50 cc. mark after Nesslerization, not, dilute with cold water.
   g. Blood obtained after a meal will give re sults 5 to 10 mg. higher than fasting blood

C. ***Koch and McMeekin's Colorimetri Method.***

1. Do test in duplicate.
2. Place 5 cc. of protein-free blood filtrate in a large dry pyrex test tube (200 by 25 mm.) graduated at 35 and 50 cc.
3. Add 1 cc. of the 1:1 sulfuric acid solution and 3 glass beads to prevent bumping. (Glass beads should be solid and 2 to 3 mm. in diameter.)
4. Heat over a microburner in a hood unti dense white fumes (SO_3) fill the tube.
5. Remove the flame and allow the tube to coo for 30 seconds to 1 minute, then add 2 drops of 30% hydrogen peroxide (superoxyl allowing it to drop directly into the solution
6. When bubbling stops, heat to boiling. If the solution has not cleared completely, repeat the addition of hydrogen peroxide.
7. Boil gently for 5 minutes.
8. Cool to room temperature and dilute to 3 cc. with water.
9. Place in an ice water bath for at least 1 minutes before Nesslerizing.
10. *Standard.*
    a. Place 5 cc. of standard ammonium sulfat solution (5 cc. = 0.15 mg. N) in a tes tube graduated at 35 and 50 cc.
    b. Add 1 cc. of 1:1 sulfuric acid solution 3 beads, and dilute to the 35 cc. mark
    c. Place in an ice water bath at the sam time as the unknowns.
11. Blow 15 cc. of Nessler's solution into eac tube using a 15 cc. pipette.
12. Insert a rubber stopper and mix by invert ing 3 times.
13. Compare immediately in a colorimeter wit the standard set at 20 mm.
14. *Calculation:*

$$\frac{RS}{RU} \times 0.15 \times \frac{100}{0.5} \doteq \frac{600}{RU} = \text{mg.\%}.$$

olin and Wu's Colorimetric Method.

o test in duplicate.
lace 5 cc. of protein-free blood filtrate in
large dry pyrex test tube (200 by 25 mm.)
raduated at 35 and 50 cc.
dd 1 cc. of sulfuric-phosphoric acid solu-
on and 3 glass beads to prevent bumping.
oil vigorously over a microburner (in
ood) until dense white fumes begin to form
t the bottom of the tube (3 to 7 minutes).
Charring will occur.
Quickly reduce the flame so the boiling prac-
ically ceases.
Cover the mouth of the test tube with a
vatch glass and continue gentle heating for
minutes; counting from the time the test
ube becomes filled with fumes. All the
rown color should disappear and the solu-
ion become faintly green or heating must
ontinue until it does.
Remove flame and allow the tube to cool
or 70 to 90 seconds.
Wash the watch glass with a few cc. of
water allowing the water to run into the tube.
Cool to room temperature and dilute to 35
cc. with water.
Place in an ice water bath for at least 15
minutes before Nesslerizing.
Standard.
a. Place 5 cc. of standard ammonium sul-
fate solution (5 cc. = 0.15 mg. N) in a
test tube graduated at 35 and 50 cc.
b. Add 1 cc. of sulfuric-phosphoric acid
solution, 3 beads, and dilute to 35 cc.
with water.
c. Place in an ice water bath at the same
time as the unknowns.
Blow 15 cc. of Nessler's solution into each
tube using a 15 cc. pipette.
Insert a rubber stopper and mix by inverting
3 times.
Compare immediately in a colorimeter with
the standard set at 20 mm.
Calculation:
See Koch and McMeekin's Method.

Photoelectric Colorimeter Method.

Digest the protein-free blood filtrate in the
same manner as described under either of
the colorimetric methods. (Do test in dupli-
cate.)
Prepare 2 standards by placing 5 cc. of the
standard ammonium sulfate solution (5 cc. =
0.15 mg. N) in each of 2 test tubes graduated
at 35 and 50 cc., add 1 cc. of the same diges-
tion solution used above to each, 3 beads, and
dilute to 35 cc. with water.
Place the standards and unknowns in an ice
water bath for 15 minutes or until the tubes have reached a temperature of 11°C.

4. Blow 15 cc. of Nessler's solution into each standard and each unknown, stopper, and mix by inversion.
5. Allow the tubes to stand for 10 minutes at room temperature, but read them within the next 10 minutes.
6. Read one of the standards as follows:
   a. Pour 10 cc. into a colorimeter tube and place in the colorimeter using filter No. 520.
   b. Adjust the galvanometer to the point at which the standard was found to read when making the calibration curve.
   c. Remove the tube and allow the galvanometer to swing back into position and take the reading.
   d. Repeat with the second standard; this reading should check that of the first standard within one division on the galvanometer.
   e. This reading corresponds to that of a blank and is used as such to read the unknowns.
7. Read each unknown and obtain the mg. per cent of nonprotein nitrogen from the table of values.
8. *Calibration of Standard Curve.*
   a. Use a standard ammonium sulfate solution in which 5 cc. = 0.15 mg. N.
   b. Place the number of cc. of the standard solution containing 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, 0.24, 0.27, 0.30, and 0.36 mg. of nitrogen serially in 10 test tubes graduated at 35 and 50 cc.
   c. Add 1 cc. of acid digestion solution (according to the method used) and 3 beads to each tube and dilute to 35 cc. with water.
   d. Prepare a blank by placing 1 cc. of the acid digestion mixture used in the test in a test tube graduated at 35 and 50 cc. and diluting to 35 cc. with water.
   e. Place all the tubes in ice water until they have reached a temperature of 11°C.
   f. Blow 15 cc. of Nessler's solution into each tube, fill to the mark with water if necessary, and mix by inversion.
   g. Allow the tubes to stand for 10 minutes at room temperature, but read them within the next 10 minutes.
   h. Pour 10 cc. of the blank solution into a colorimeter tube, and with this tube adjust the galvanometer to 100 using filter No. 520.
   i. Remove the tube, record the galvanometer reading, and then read each of the standards.

  j. Perform a sufficient number of determinations on each concentration of nitrogen that an average of the readings obtained will give a straight line when plotted on semilogarithmic graph paper.
  k. Make a table of nitrogen values for each galvanometer division.
  l. Convert the nitrogen to mg. per cent of nonprotein nitrogen by multiplying by 200 because a 1-200 dilution of blood is used in the test.

F. *Solutions.*

1. *Sulfuric Acid Digestion Solution* (1:1)—Mix 1 part of c.p. conc. sulfuric acid (free from the least trace of ammonia) with 1 part of water.
2. *Sulfuric-Phosphoric Acid Digestion Solution* for Folin and Wu's Method.
    - a. To 50 cc. of a 5% copper sulfate ($CuSO_4 \cdot 5H_2O$) solution in a 1 liter Erlenmeyer flask, add 300 cc. of 85% phosphoric acid and mix. The copper sulfate acts as a catalyst.
    - b. Add 100 cc. of conc. sulfuric acid free from the least trace of ammonia and mix.
    - c. Stopper tightly and set aside several days for any calcium sulfate to settle.
    - d. Pour the clear supernatant fluid into a 500 cc. graduated cylinder and measure.
    - e. Slowly pour this clear acid mixture into an equal amount of water and mix thoroughly.
    - f. One cc. of this solution should require 9 to 9.3 cc. of Nessler's solution to neutralize it.
    - g. Keep well stoppered to prevent absorption of ammonia from the air.
3. *Nessler's Reagent.*
    - a. This is an alkaline solution of the double iodide of mercury and potassium iodide ($HgI_2 \cdot 2KI$).
    - b. To 150 gm. of potassium iodide and 100 gm. of iodine in a 500 cc. Florence flask, add 100 cc. of water and an excess of metallic mercury, 140 to 150 gm.
    - c. Shake continuously and vigorously until the iodine has nearly all disappeared (7 to 15 minutes). The solution becomes quite hot.
    - d. When the red iodine solution becomes visibly pale though still red, cool in running water and continue shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide.
    - e. Decant the solution into a 2 liter volumetric flask.
    - f. Wash the mercury and flask with liberal quantities of distilled water and add washings (provided they are clear) to the decanted solution.
    - g. Dilute the solution and washings to 2 liters and mix well.
    - h. If clear, this solution may be used at once for dilution to Nessler's solution; if not clear, let stand one week before diluting the supernatant fluid.
4. *Nessler's Solution.*
    - a. Place 750 cc. of the clear Nessler's reagent and 750 cc. of water in a 5 liter flask.
    - b. Add 3500 cc. of 10% NaOH a little at a time with vigorous shaking.
        1) The 10% NaOH is made by diluting 600 cc. of a saturated solution (110 gm. NaOH pellets to 100 cc. water) to 4 liters. The saturated solution should be allowed to stand until the carbonate has settled, the clear supernatant solution being decanted and used.
        2) This 10% solution (2.5 N) must be standardized accurately by titrating against N hydrochloric acid or oxalic acid. Ten cc. of the 10% NaOH should neutralize 25 cc. of N acid using alizarin red as an indicator.
    - c. The alkalinity of the Nessler's solution is important. Titrate 20 cc. portions of N HCl with the Nessler's solution. A good end-point with phenolphthalein should be obtained at 11 to 11.5 cc. If as low as 9.5 cc., the solution is too alkaline and should be discarded.
    - d. Nessler's solution should be used in the ratio of 10 cc. per 100 cc. of solution to be Nesslerized, except when excessive amounts of acid are present as in direct Nesslerization procedures.
5. *Standard Ammonium Sulfate Solution.*
    - a. *Stock Standard Solution* (1 cc. = 2 mg. N).
        1) Ammonium sulfate, c.p. [$(NH_4)_2SO_4$, special "pyridine free"] is dried by heating in a 100°C. oven for 1 hour, then placed in a desiccator overnight.
        2) Weigh exactly 1.887 gm. and place in a 200 cc. volumetric flask.
        3) Dilute to volume with 0.2 N sulfuric acid.
    - b. *Dilute Standard Solution* (5 cc. = 0.15 mg. N).
        1) Pipette 15 cc. of the stock standard solution into a liter volumetric flask.
        2) Dilute to volume with 0.2 N sulfuric acid.

erpretation of Blood Nonprotein Nitro-n Findings.

rmal Values: 25—35 mg. per cent.

reased due to:

Renal impairment.
Early nephritis (35—50 mg. %)
Severe glomerulonephritis (50 mg. % and up)
Late stages of arteriolonephrosclerosis with hypertension
Uremia (may be high as 400 mg. %)
Urinary obstruction
Metallic poisoning, especially $HgCl_2$

Augmented N catabolism.
Febrile infections
Hemorrhage in the upper G.I. tract
Intestinal obstruction
Acute generalized peritonitis
Dehydration
Hyperemesis
Leukemia
Gout
Eclampsia

. *Other causes.*
Cardiac decompensation
Coronary thrombosis
Acute yellow atrophy of the liver
Addison's disease in crisis

ecreased in:
ormal pregnancy (slight)
iabetes mellitus (normal or lowered)
iabetes insipidus

. Total Protein, Albumin, Globulin, and Fibrinogen (Modified Howe's Method).

Principle: Total proteins are determined in serum or in plasma by a micro-Kjeldahl method. They are converted to ammonium sulfate and the nitrogen determined by Nesslerization, the nonprotein nitrogen being taken into consideration. Globulin is precipitated out of serum by sodium sulfate and the albumin is determined on the filtrate. The fibrinogen is removed from the plasma by treating with calcium chloride and its nitrogen content determined.

General Considerations.

Blood should be collected within 1 minute after the application of the tourniquet and the tourniquet should be released after entering the vein.
Hemolysis of the blood must be avoided.
A total protein determination on serum does not include fibrinogen.
A fibrinogen determination must be done on plasma.

5. *Sources of error.*
   a. If the tubes are not heated slowly, the liquid will boil above the 35 cc. mark and the results will be low.
   b. Incomplete precipitation of globulin will give a high albumin value.
   c. Careless pipetting of 1 cc. portions.
   d. Distilled water used in the test must be free of any nitrogen compounds.

C. *Colorimetric Method.*

1. *Total Protein (T.P.).*
   a. Pipette 1 cc. of serum into a 50 cc. volumetric flask and dilute to volume with 0.85% sodium chloride solution.
   b. Mix and transfer 1 cc. to a pyrex test tube graduated at 35 and 50 cc. (do in duplicate).
   c. Add 1 cc. of 1:1 sulfuric acid solution and 3 beads.
   d. Digest by heating over a microburner in a hood until dense white fumes (SO_3) fill the tube.
   e. Remove the flame and allow the tube to cool for 30 seconds to 1 minute, then add 2 drops of 30% hydrogen peroxide (superoxyl) allowing it to drop directly into the solution.
   f. When bubbling stops, heat to boiling. If the solution has not cleared completely, repeat the addition of hydrogen peroxide.
   g. Boil gently for 5 minutes.
   h. Remove tube from flame and cool.
   i. Continue under Nesslerization, step 5.
2. *Albumin* (Kingsley's Method).
   a. Place 30 cc. of 23% sodium sulfate solution in a 50 cc. centrifuge tube without a lip.
   b. Add 1 cc. of serum, stopper, and mix by inverting several times.
   c. Add 8 cc. of ethyl ether, stopper, and slowly invert twice.
   d. Take out stopper for a second, then replace, and shake *vigorously* for 30 seconds.
   e. Remove stopper and centrifuge for 10 minutes at 2200 revolutions per minute.
   f. Slant the tube so that the tightly packed globulin layer floating on the sodium sulfate solution is separated from the walls of the tube, insert a volumetric 10 cc. pipette through the ether layer along the lower wall of the tube, remove approximately 10 cc. of the clear fluid. Place the fluid in a test tube leaving the last cc. in the pipette to be discarded in case any ether was drawn up into the pipette.

g. Pipette two 1 cc. portions into each of 2 pyrex test tubes graduated at 35 and 50 cc.
h. Add 3 beads and 1 cc. of 1:1 sulfuric acid solution to each tube.
i. Digest as for total protein.
j. Continue under Nesslerization, step 5.

3. *Fibrinogen.*
   a. Do test in duplicate.
   b. Place 30 cc. of 0.85% sodium chloride solution in a large tube. Add 1 cc. of plasma and 1 cc. of 2.5% calcium chloride solution.
   c. Stopper, mix by repeated inversion, and allow to stand undisturbed for 30 minutes to 1 hour.
   d. Loosen the clot from the walls of the test tube with a slender glass rod with a pointed tip and filter the contents through a large Whatman No. 50 filter paper.
   e. When most of the sodium chloride solution has filtered off, remove the filter paper and lay out flat on several thicknesses of filter paper to drain.
   f. Wind the clot around the stirring rod being careful to collect all of the fibrinogen.
   g. Dry the clot between filter paper and place it in a pyrex test tube graduated at 35 and 50 cc.
   h. Add 3 beads and 1 cc. of 1:1 sulfuric acid solution.
   i. Digest as for total protein above.
   j. Continue under Nesslerization, step 5.
4. *Standard.*
   a. Place 5 cc. of ammonium sulfate standard solution (5 cc. = 0.2 mg. N) in a test tube graduated at 35 and 50 cc.
   b. Add 3 beads and 1 cc. of 1:1 sulfuric acid solution.
5. *Nesslerization.*
   a. Dilute unknowns (total protein, albumin, and fibrinogen) and standard to 35 cc. with water and place in an ice water bath for at least 15 minutes.
   b. Blow 15 cc. of Nessler's solution into each using a 15 cc. pipette.
   c. Insert a rubber stopper and mix by inverting 3 times.
   d. Compare in a colorimeter with the standard set at 20 mm.
6. *Calculation:*
   a. *Total Protein Nitrogen.*

$$\frac{RS}{RU} \times 0.2 \times \frac{100}{0.02} = \text{mg. \% of N.}$$

   b. *Albumin Nitrogen.*

$$\frac{RS}{RU} \times 0.2 \times \frac{100}{0.0323} = \text{mg. \% of N.}$$

   c. *Fibrinogen Nitrogen.*

$$\frac{RS}{RU} \times 0.2 \times 100 = \text{mg. \% of N.}$$

   d. *Conversion of Nitrogen to Protein.*
      1) The nitrogen of the total protein an albumin includes the nonprotein nitrogen which must be subtracted. If th nonprotein nitrogen was not determined, subtract an average value of 3 mg.
      2) Since 1 gm. of nitrogen represents 6.25 gm. of protein, transform each of the above to protein by the following formula, except fibrinogen which does not have the nonprotein nitrogen subtracted.

$$\frac{\text{mg.N} - \text{mg. N P N}}{1000} \times 6.25 = \text{gm.\% of protein.}$$

   e. *Globulin* = gm. of Total Protein — gm. of Albumin.

D. *Photoelectric Colorimeter Method.*

1. Follow directions under colorimetric method for total protein, albumin, and fibrinogen to Nesslerization, except prepare 2 standards instead of one.
2. Nesslerize and read as described under the photoelectric colorimeter method for nonprotein nitrogen.
3. The table of mg. of nitrogen on which the nonprotein nitrogen values were based is used in the following calculations:
   a. *Total Protein.*

$$\frac{(\text{mg. N} \times 5000) - \text{N P N}}{1000} \times 6.25 = \text{gm. \% of protein.}$$

   b. *Albumin.*

$$\frac{(\text{mg. N} \times 3100) - \text{N P N}}{1000} \times 6.25 = \text{gm. \% of albumin.}$$

   c. *Fibrinogen.*

$$\frac{(\text{mg. N} \times 100)}{1000} \times 6.25 = \text{gm. \% of fibrinogen.}$$

   d. *Globulin* = gm. of Total Protein — gm. of Albumin.

E. *Solutions.*

1. *Sodium Sulfate Solution—23%.*
   a. Place 230 gm. of anhydrous sodium sulfate in a liter volumetric flask and add about 700 cc. of hot water.
   b. When the sodium sulfate is dissolved, cool to 20°C. and dilute to volume with water.
   c. Using a dilute solution of phenol red as an indicator, adjust the pH to a faint pink with sodium hydroxide or sulfuric acid.

lcium Chloride Solution—2.5%.

Place 2.5 gm. of anhydrous $CaCl_2$ in a 100 cc. volumetric flask.

Make up to volume with water.

ndard Ammonium Sulfate Solution (5 cc. 0.2 mg. N).

See standard solution under nonprotein nitrogen determination for directions for making stock standard solution.

Place 10 cc. of the stock standard solution (1 cc. = 2 mg. N) in a 500 cc. volumetric flask. Dilute to volume with 0.2 N sulfuric acid.

ther solutions are described under N P N ethod.

terpretation of Blood Protein Findings.

ormal Values:

otal proteins: 6—8 gm. per cent.

lbumin: 3.5—5.6 gm. per cent.

lobulin: 1.3—3.2 gm. per cent.

lbumin-globulin ratio 1.5:1 to 3:1.

ibrinogen: 0.2—0.4 gm. per cent.

bnormal Values: See Table 80.

dema occurs when the serum protein level lls below 5 gm. per cent.

III. Spinal Fluid Total Protein.

rinciple: The protein is precipitated from e spinal fluid with acid tungstate, disolved, and reprecipitated. The nitrogen is etermined on an aliquot portion after being ut into solution with the aid of sodium ydroxide.

eneral Considerations.

resence of blood gives false high values.

resence of bacteria gives unreliable results.

elay of analysis increases the value obtained nless fluid is kept sterile and tightly corked.

Colorimetric Method.

Place 3 cc. of spinal fluid in a graduated centrifuge tube and add 3 cc. of water. (Use 1 cc. of spinal fluid if protein is high.)

Add 1.2 cc. of 2/3 N sulfuric acid and 1.2 cc. of 10% sodium tungstate solution.

Stopper and mix by inverting; remove stopper and save for later mixings.

Centrifuge and decant clear supernatant fluid. (The supernatant fluid may be used for sugar determination.)

Add 0.6 cc. of 10% sodium tungstate solution to the precipitate and mix until all of the protein is dissolved.

Add 6 cc. of water and 0.6 cc. of 2/3 N sulfuric acid.

7. Stopper to mix and then centrifuge.
8. Decant supernatant fluid. Add 1 drop of approximately 5 N NaOH to the precipitate.
9. Shake until the protein is dissolved.
10. Make up to 3 cc. with water, stopper, and mix.
11. Place 1 cc. of the above in a pyrex test tube marked at 35 and 50 cc. (do in duplicate).
12. Add 3 beads and 1 cc. of 1:1 sulfuric acid solution.
13. Digest as for total protein (p. 275).
14. *Standard.*
    a. Place 3 cc. of ammonium sulfate standard solution (3 cc. = 0.09 mg. N) in a test tube marked at 35 and 50 cc.
    b. Add 3 beads and 1 cc. of 1:1 sulfuric acid solution.
15. Dilute unknown and standard to 35 cc. with water and place in an ice water bath for at least 15 minutes.
16. Blow 15 cc. of Nessler's solution into each tube.
17. Insert a rubber stopper and mix by inverting 3 times.
18. Compare in a colorimeter with the standard set at 20 mm.
19. *Calculation:*
    a. When 3 cc. of the standard is used:

$$\frac{RS}{RU} \times 0.09 \times \frac{3}{\text{cc. sp. fl. used}} \times 100 \times 6.25 = \text{mg. \% protein.}$$

    b. When 2 cc. of the standard is used:

$$\frac{RS}{RU} \times 0.06 \times \frac{3}{\text{cc. sp. fl. used}} \times 100 \times 6.25 = \text{mg. \% protein.}$$

D. Photoelectric Colorimeter Method.

1. Follow directions under colorimetric method to the digestion (13 above).
2. Digest as for total proteins.
3. Continue as described under the photoelectric colorimeter method for N P N using 3 cc. of the standard solution (3 cc. = 0.09 mg. N) and setting the galvanometer accordingly.
4. The table of mg. of nitrogen on which the N P N values were based is used in the following calculation:

$$\text{mg.N} \times \frac{3}{\text{cc. sp. fl. used}} \times 100 \times 6.25 = \text{mg. \% of protein.}$$

E. Solutions.

1. *Sulfuric Acid Solution*—2/3 N, see page 264.
2. *Sodium Hydroxide Solution*—5 N.
    a. Place 27.5 cc. of the supernatant fluid from a stock NaOH (110 gm. of NaOH

TABLE 80. SIGNIFICANCE OF BLOOD PROTEIN FINDINGS*

Pathological Contition	Total Protein	Albumin	Globulin	Fibrinog
Anemias (severe)	Dec.	Dec.	Dec.	Dec.
Carcinoma	N to Inc.	N to Dec.	N to Inc.	Inc.
Dehydration, rapid with hemoconcentration (severe diarrhea and vomiting, restriction of fluids, high intestinal obstruction, shock, extensive burns, Addison's disease in crisis)	***Inc.***	Inc.	Inc.	Inc.
Dehydration, slow without hemoconcentration (prolonged vomiting or diarrhea, severe burns, and sprue)	***Dec.***	***Dec.***	N	N
Edema				
Nephritic	Dec.	***Dec.***	N to Inc. (R)	***Inc.***
Nutritional and Beriberi	***Dec.***	***Dec.***	N	N
Cardiac	N to Dec.	N to Dec.	N	N
Glomerulonephritis				
Acute	***Dec.***	***Dec.***	N to Inc.	***Inc.***
Chronic	***Dec.***	***Dec.***	N to Inc. (R)	N
Hemochromatosis	***Dec.***	***Dec.***	Inc. (R)	Dec.
Hemorrhage (severe)	Dec.	Dec.	N to Dec.	Dec.
Infections				
Infections accompanied by leukocytosis or suppuration	N to Dec.	N to Dec.	Inc.	Inc.
Pneumonia	N to Inc.	N to Dec.	Inc.	Inc.
Typhoid fever	Dec.	Dec.	N	Dec.
Tuberculosis	N to Inc.	N	Inc.	Inc.
Leprosy	N to Inc.	Dec.	Inc.	
Typhus fever	Dec.	Dec.	***Inc.***	
Lymphogranuloma venereum	Inc.	N	***Inc.***	
Syphilis	Inc.	N	Inc.	
Leukemia	Dec.	Dec.	Inc.	Dec.
Liver disease				
Cirrhosis with ascites	***Dec.***	***Dec.***	N to Inc. (R)	Dec.
Infectious hepatitis	Dec.	Dec.	***Inc.***	Inc.
Parenchyma destruction	Dec.	Dec.	N	***Dec.***
Malnutrition	***Dec.***	***Dec.***	N	N
Multiple myeloma	***Inc.***	N to Dec.	***Inc.***	Inc.
Nephrosis	***Dec.***	***Dec.***	N to Inc. (R)	***Inc.***
Parasitic infections				
Kala-azar	N to Inc.	Dec.	***Inc.***	Inc.
Malaria	Dec.	Dec.	Inc.	N
Schistosomiasis	N to Inc.	Dec.	***Inc.***	N
Pregnancy with toxemia	Dec.	Dec.	N to Inc. (R)	Inc.
Sarcoidosis	Inc.	Dec.	***Inc.***	N

*The Dec. or Inc. in bold face italics means the decrease or increase is of diagnostic significance. N means normal (R) means the increase is relative.

pellets per 100 cc. of water) solution in a 100 cc. volumetric flask.

b. Dilute to volume with water.

3. *Standard Solution*—same as used for N P N.

F. *Interpretation of Spinal Fluid Total Protein Findings.*

1. See Table 26 on page 154.

2. An increase as high as 300 mg. per cent may be obtained in myxedema.

3. An increase is sometimes obtained in arthritis of the spine.

IV. Urea Nitrogen (B U N)
(Folin and Svedberg's Method).

A. ***Principle:*** Urea is hydrolized to ammonium

rbonate by means of the enzyme urease
the presence of a buffer solution (pH
8). The ammonia is liberated by the addi-
on of the sodium borate and then distilled
to 0.05 N hydrochloric acid. The amount
nitrogen present is determined colori-
etrically after Nesslerization.

ources of Error.

oo much oxalate or citrate as an antico-
ulant interferes with the urease reaction.

lassware that has had Nessler's solution in
must be washed with nitric acid before
can be used for the urease reaction.

ubes that have contained cyanate should
ever be used for urea determinations.

xcess buffer interferes with the urease re-
ction.

he urea will be increased if too much
rease is used.

oo long heating during distillation.

Colorimetric Method.

Place 5 cc. of protein-free blood filtrate in an unmarked pyrex tube (20x200 mm.).

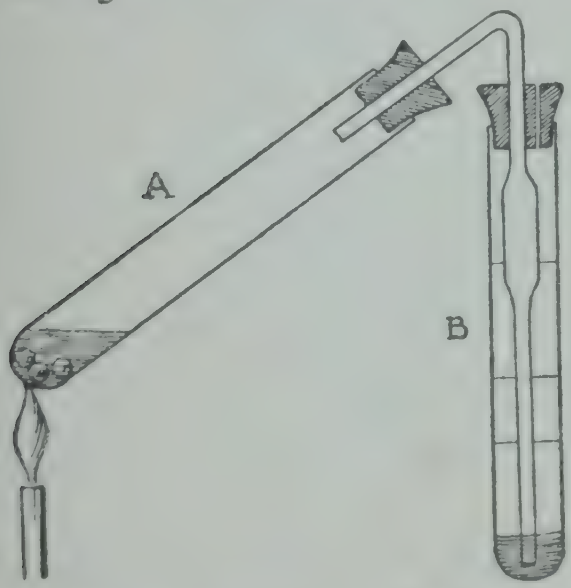
Add 6 drops of acetate buffer solution or 2 drops of sodium pyrophosphate buffer.

Add 1 cc. of urease solution.

Immerse the tube in a 55°C. water bath for 15 minutes. The temperature should not drop below 45°C.

Cool the tube, add 3 beads and allow 4 drops of mineral oil to run down the inside of the tube.

Place 2 cc. of 0.05 N HCl in a pyrex test tube graduated at 25, 35, and 50 cc. (tube B in Fig. 28) and insert the delivery tube.



28. Distilling apparatus for the determination of blood urea nitrogen.

a. Tube B must have a bore of such size that a slit cut in the rubber stopper will remain open to allow the escape of steam.

b. The delivery tube is a 5 or 10 cc. volumetric pipette bent at a 45° angle and it must be long enough to extend below the surface of the HCl in tube B.

7. Blow 2 cc. of saturated sodium borate solution into tube A containing the filtrate and immediately connect to the delivery tube.
8. Boil the mixture of filtrate and borate gently with a low flame for 1 minute, being careful that the distillate does not suck back.
9. Increase the heat and boil until steam begins to escape from the slit in the stopper of tube B which takes about 5-7 minutes, then continue heating for 1 minute. Prolonged heating will cause false high results.
10. Disconnect tube B and heat tube A one minute with the delivery tube slightly above the surface of the liquid.
11. Rinse the lower end of the delivery tube and the sides of tube B with a little water, and dilute with water until the total volume is about 20 cc.
12. Place in an ice water bath for at least 15 minutes before Nesslerizing. When there is more than 1 determination, set each aside until all are cooked, then place all the tubes and 1 standard in the water bath at the same time.
13. *Standard.*
    a. Place 5 cc. of ammonium sulfate standard solution (5 cc. = 0.15 mg. N) in a tube marked at 50 cc.
    b. Add 35 to 40 cc. of water and cool in an ice water bath with the unknowns.
14. Blow 5 cc. of Nessler's solution into the tube containing the standard, dilute to 50 cc., mix, and standardize the colorimeter.
15. Blow 2.5 cc. of Nessler's solution into each unknown, dilute to 25 cc. and mix. If the color is dark, add an additional 2.5 cc. of Nessler's solution and dilute to 50 cc.
16. Compare in colorimeter with the standard set at 20 mm.
17. *Calculation:*

$$\frac{RS}{RU} \times 0.15 \times \frac{100}{0.5} \times \frac{25}{50} = \frac{300}{RU} = \text{mg.\%}$$

D. *Photoelectric Colorimeter Method.*

1. Follow colorimetric method to the point of Nesslerization, except prepare 2 standards instead of one.
2. Place the tubes in ice water until they have reached a temperature of 11°C.
3. Blow 5 cc. of Nessler's solution into each of the standards, dilute to 50 cc. with cold water, and mix.
4. Blow 2.5 cc. of Nessler's solution into each

unknown, dilute to 25 cc. with water, and mix.

5. Allow the tubes to stand for 15 minutes at room temperature, but read them within the next 10 minutes.
6. Read one of the standards as follows:
   a. Pour 10 cc. into a colorimeter tube and place in the colorimeter using filter No. 520.
   b. Adjust the galvanometer to the point at which the standard was found to read when making the calibration curve.
   c. Remove the tube and allow the galvanometer to swing back into position and take the reading.
   d. Repeat with the second standard; this reading should check that of the first standard within 1 division on the galvanometer.
   e. This reading corresponds to that of a blank and is used as such to read the unknowns.
7. Read each unknown and obtain the mg. per cent of urea from the table of values.
8. If the value is over 30 mg. per cent, dilute the filtrate with water and repeat the determination.

9. *Calibration of Standard Curve.*
   a. Use a standard ammonium sulfate solution in which 5 cc. = 0.15 mg. N.
   b. Place the number of cc. of the standard solution containing 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, 0.24, 0.27, 0.30, and 0.36 mg. of nitrogen serially in 10 test tubes graduated at 50 cc.
   c. Dilute each tube to about 40 cc. with water.
   d. Prepare a blank tube of about 40 cc. of water.
   e. Place all the tubes in ice water until they have reached a temperature of 11°C.
   f. Blow 5 cc. of Nessler's solution into each tube, fill to the mark with water, and mix by inversion.
   g. Allow the tubes to stand 15 minutes at room temperature, but read them within the next 10 minutes.
   h. Pour 10 cc. of the blank solution into a colorimeter tube, adjust the galvanometer to 100 using filter No. 520.
   i. Remove the tube, record the galvanometer reading, and then read each of the standards.
   j. Perform a sufficient number of determinations on each concentration of nitrogen that an average of the readings obtained will give a straight line when plotted on semilogarithmic graph paper.
   k. Make a table of values for urea nitrogen mg. per cent by multiplying the mg. N 100 (0.01 mg. N. is equivalent to 1 m per cent of urea nitrogen). The ta should not be higher than 30 mg. per ce because values above this are inaccura and the test should be repeated us filtrate that has been diluted with wate

E. *Solutions.*

1. *Buffer Solutions.*
   a. *Acetate Buffer.*
      1) Place 9 gm. of anhydrous sodiu acetate ($NaC_2H_3O_2$) or 15 gm. o crystalline sodium acetate (NaC_2H_3O $\cdot$ $3H_2O$) in a 100 cc. volumetric flas and dissolve in about 50 cc. of water
      2) Add 1 cc. of glacial acetic acid an dilute to volume with water.
   b. *Sodium Pyrophosphate Buffer.*
      1) Place 140 gm. sodium pyrophosphat ($Na_4P_2O_7 \cdot 10H_2O$) in a liter volumetric flask.
      2) Add 0.5 N phosphoric acid to volume.
      3) The 0.5 N phosphoric acid is mad as follows:
         a) Dilute 20 cc. of 85% orthophosphoric acid to 1 liter with water
         b) Titrate 5 cc. of this acid with 0. N NaOH using phenolphthalei as an indicator.
         c) Five cc. of the acid should neutralize 18 cc. of 0.1 N NaOH.
2. *Hydrochloric Acid*—0.05 N.
   a. Add 5 cc. of conc. hydrochloric acid t about 700 cc. of water in a liter volumetric flask and dilute to volume wit water.
   b. Mix well and standardize with 0.1 N NaOH using phenolphthalein as an indicator.
   c. Twenty cc. of 0.05 N HCl is neutralize by 10 cc. of 0.1 N NaOH.
3. *Saturated Sodium Borate Solution*—dissolv 60 gm. of pure sodium tetraborate (Na_2B_4O $\cdot$ $10H_2O$) in 500 cc. of hot water.
4. *Urease.*
   a. Place 3 gm. of permutit in a 250 cc. flas with 100 cc. of 2% acetic acid.
   b. Shake vigorously, let stand for a fe minutes, and then decant the supernatan fluid.
   c. Wash the residue 2 or 3 times with 1 cc. portions of water.
   d. Add 100 cc. of 15% ethyl alcohol (cc. of 95% alcohol diluted to 100 cc. w water) and 5 gm. of jack bean meal.

Shake vigorously and continuously for 15 minutes.

Filter in the refrigerator through Whatman No. 40 filter paper. Cover the funnel with a watch glass.

The solution will keep in the refrigerator about 1 month.

. Check the new solution of urease and again at weekly intervals by using 5 cc. of a 0.004% urea solution instead of the protein-free filtrate in the test for urea N.
  1) The 0.004% solution is made by placing 1 cc. of a 0.2% urea solution in a 50 cc. volumetric flask and diluting to volume with water.
  2) This should read 16 or 16.1 mm. on the colorimeter or equal 18.66 mg. urea N.

For other solutions see method for nonrotein nitrogen.

Interpretation of Blood Urea Findings.

Normal Values: 10—15 mg. per cent.

a. The concentration of blood urea is influenced by the amount of protein metabolism and the rate of excretion.

b. It tends to be high when protein metabolism is increased by diet and fever and is somewhat diminished when protein metabolism is at a low level.

c. Normally it is approximately one half of the nonprotein nitrogen, but in retention it may constitute 80 to 90 per cent.

Increase due to:

a. *Renal impairment.*
   - Nephritis—acute and chronic
   - Metallic poisoning
   - Double polycystic kidney and urinary obstruction

b. *Augmented nitrogen catabolism.*
   - Intestinal obstruction
   - Dehydration
   - Pneumonia
   - Acute generalized peritonitis
   - Bleeding peptic and duodenal ulcers due to digestion of erythrocytes in the upper intestine; also, in bleeding carcinoma of the stomach and in bleeding esophageal varices in cirrhosis of the liver.

c. *Other causes.*
   - Addison's disease
   - Surgical shock

Decreased in:

Acute hepatic insufficiency
Nephrosis
Chronic wasting diseases
Amyloidosis
Pregnancy

V. Urea Clearance Test (Moller, McIntosh, and Van Slyke).

A. ***Principle:*** The clearance of a substance by the kidney is the minimum volume of blood required to furnish the quantity of that substance excreted in the urine in one minute. Urea passes freely into the glomerular filtrate in the same concentration as it occurs in blood plasma; a portion of it is reabsorbed by the tubules; and the remainder is excreted in the urine. The percentage of reabsorbed urea decreases as the urine flow increases. The urine flow per minute is determined from the quantity of urine excreted in a known time. The urea clearance is UV/P when U is the urine urea N, V is the volume of urine per minute, and P is the plasma urea N. This clearance is corrected to the average body surface area of 1.73 sq. meters and reported in per cent of an established normal for the cc. of urine flow per minute found in the test.

B. ***General Considerations.***

1. Exercise preceding the test should be avoided because it decreases the clearance.
2. Patients with nephritis should be kept in bed during the test.
3. Administration of ammonium salts of organic acids must be avoided.
4. Small doses of adrenalin increase, while pituitrin and large doses of adrenalin decrease the clearance.

C. ***Collection of Specimens.***

1. The test is performed during the morning hours after a light breakfast without tea, coffee, or milk.
2. The patient is given 1 glass of water at the beginning of the test which should be at least 1 hour after breakfast. No fluid should be taken from breakfast to this time.
3. The patient then voids urine and the exact time recorded. In the performance of this test a stop watch is very useful.
4. Blood for the urea determination is drawn 1 hour later.
5. Urine is collected at the end of 2 hours; the exact time at the end of the collection is noted.
6. The volume is measured carefully and the number of cc. of urine excreted per minute is calculated.

D. ***Plasma Urea Nitrogen.***

1. Determine the urea N on plasma as described under urea nitrogen on page 278.
2. Make the filtrate according to the method for plasma filtrates on page 265.

E. *Urine Urea Nitrogen* (Colorimeter Method).

1. Dilute the urine according to the volume of urine excreted in the 2 hour period.
   a. A 1—10 dilution if the volume is under 100 cc.
   b. A 1—5 dilution if the volume is over 100 cc.
2. Mix well and place 20—30 cc. of the diluted urine in a 200 cc. Erlenmeyer flask.
3. Add 4—5 gm. of permutit and shake gently for 5 minutes to remove the ammonia.
4. Allow to settle for 30 seconds and then filter through double Whatman No. 50 filter paper until clear.
5. Place 1 cc. in a large test tube with a lip, add 6 drops of acetate buffer or 2 drops of sodium pyrophosphate buffer and 1 cc. of urease.
6. Place 2 cc. in another large test tube with a lip and add 12 drops of acetate buffer or 4 drops of sodium pyrophosphate buffer and 2 cc. of urease.
7. Incubate both tubes in a water bath between 45 and 55°C. for 15 minutes.
8. Transfer each of the incubated urines quantitatively to 200 cc. volumetric flasks and dilute to 170—175 cc. with distilled water.
9. *Standard.*
   1) Place 5 cc. (0.3 mg. N) of ammonium sulfate solution in a 100 cc. volumetric flask, add 3 drops of acetate buffer or 1 drop of sodium pyrophosphate buffer, and 0.5 cc. of urease solution.
   2) Place 10 cc. (0.6 mg. N) of ammonium sulfate solution in a 100 cc. volumetric flask, add 6 drops of acetate buffer or 2 drops of sodium pyrophosphate buffer, and 1 cc. of urease solution.
   3) Dilute each to 70—80 cc. with water.
10. Cool the unknowns and the standards in an ice water bath for at least 15 minutes.
11. Blow 10 cc. of Nessler's solution into each standard.
12. Blow 20 cc. of Nessler's solution into each unknown.
13. Dilute to volume with cold water and compare the unknown in the colorimeter with the standard that most closely approximates its color.
14. Set the standard at 20 mm.
15. *Calculation:*

$$\frac{RS}{RU} \times St. \times \frac{100}{cc.\ urine^*} = mg.\ \%.$$

*0.1 or 0.2 cc. if 1—10 dilution.
0.2 or 0.4 cc. if 1—5 dilution.

16. Normal urea N excretion in 24 hours is 7 to 15 gm. depending upon the amount of protein in the diet. To change urea N to urea multiply by 2.14. The normal urea content is 15 to 35 gm.

F. *Urine Urea Nitrogen* (Photoelectric Colorimeter Method).

1. Follow the colorimeter method up to the addition of Nessler's solution, except make 2 standards using 5 cc. of the standard ammonium sulfate solution (0.3 mg. N).
2. Place the flasks containing the unknown and standards in an ice water bath until they have reached a temperature of 11°C.
3. Blow 10 cc. of Nessler's solution into each standard, dilute to volume with water, and mix.
4. Blow 20 cc. of Nessler's solution into the unknown, dilute to volume with water, and mix.
5. Allow the flasks to stand 15 minutes at room temperature, but read them within the next 10 minutes.
6. Using filter No. 520, set the galvanometer with the standard and read the unknowns according to the directions under blood urea determination.
7. *Calculation:*
   a. Obtain the mg. of nitrogen for the galvanometer reading from the table of values for urea nitrogen by dividing by 100.

$$Mg.\ N \times 4 \times 100 \times \frac{dilution\ of\ urine}{cc.\ of\ diluted\ urine\ used} =$$

mg. % of urea N.

The 4 is used in the calculation because the nitrogen values are based on a final volume of 50 cc. instead of 200.

G. *Urea Clearance Calculation.*

1. *Method Using Surface Area of Patient.*
   a. Obtain weight and height of patient and calculate surface area.

$S.A. = Wt.^{0.425} \times Ht.^{0.725} \times 71.84.$
S.A. = surface area in sq. cm. (divide by 10,000 to obtain square meters).
Wt. = weight in kg.
Ht. = height in cm.

   b. Calculate urine flow in cc. per minute.
   c. Clearance:

$$\frac{Urine\ urea\ N \times cc.\ urine\ per\ minute}{Plasma\ urea\ N}$$

= cc. of plasma cleared per minute.

   d. Correct cc. of plasma cleared per minute to the average surface area of 1.73 sq meters.

Formula:

$$\frac{1.73}{\text{S. A. of patient}} \times \text{cc. plasma cleared per minute.}$$

The percentage of normal function for any urine flow is determined by dividing the corrected urea clearance by the normal value for that urine flow as given in Table 81.

LE 81. NORMAL UREA CLEARANCE VALUES

Urine Flow cc./min.	Urea Clearance cc./1.73 sq. M/min.
0.10	9.0
0.20	18.0
0.30	27.0
0.40	36.0
0.50	43.0
0.75	50.0
1.00	54.0
1.50	57.0
2.00	60.0
4.00	62.5
6.00	65.0
8.00	66.5
10.00	68.0
12.00	73.0
14.00	78.0

Method Using Standard, Maximum, and Minimum Clearance.

. *Standard clearance*—Used when urine flow is between 0.35—2 cc. per minute.

$$\frac{\sqrt{\text{Urine per min.}} \times \text{Urine urea N}}{\text{Plasma urea N}}$$

= cc. of plasma cleared per minute.

1) *Normal standard clearance* of plasma per minute is 54 cc. with a range from 40—68 cc.

2) *Per cent of normal clearance* = cc. of plasma cleared × 1.85. (The 1.85 is obtained by dividing 100 by 54.)

b. *Maximum clearance*—Used when urine flow is more than 2 cc. per minute.

$$\frac{\text{Urine per min.} \times \text{Urine urea N}}{\text{Plasma urea N}}$$

= cc. of plasma cleared per minute.

1) *Normal maximum clearance* of plasma per minute is 75 cc. with a range from 64—99 cc.

2) *Per cent of normal clearance* = cc. of plasma cleared × 1.33. (The 1.33 is obtained by dividing 100 by 75.)

c. *Minimum clearance*—Used when urine flow is less than 0.35 cc. per minute.

$$\frac{\text{Urine urea N}}{\text{Plasma urea N}} = \text{cc. of plasma cleared per minute.}$$

1) *Normal minimum clearance* is 32 cc. of plasma per minute with a range of 24—40 cc.

2) *Per cent of normal clearance* = cc. of plasma cleared × 3.12. (The 3.12 is obtained by dividing 100 by 32.)

H. *Solutions.*

1. *Standard Ammonium Sulfate Solution* (5 cc. = 0.3 mg. N).
   a. Place 15 cc. of the stock ammonium sulfate standard solution (1 cc. = 2 mg. N) in a 500 cc. volumetric flask.
   b. Dilute to volume with 0.2 N sulfuric acid.
   c. For directions to make stock standard solution see page 274.
2. For other solutions see method for blood urea nitrogen.

I. *Interpretation of Urea Clearance Findings.*

1. Normal clearance is 100 per cent (ranges from 60—125 per cent).
2. Figures below 60% imply decreased kidney efficiency.
   a. Mild impairment: 40—60 per cent.
   b. Moderate impairment: 20—40 per cent.
   c. Severe impairment: Less than 20 per cent.
3. May be decreased due to changes in blood urea N from causes other than kidney damage.
   a. See increase in urea N due to augmented N catabolism on page 281.
   b. See decrease in urea N on page 281.
4. In patients with diminishing renal function, the urea clearance usually falls before there is an increase in blood urea.
5. Blood uric acid may be elevated before abnormal clearance values are obtained.
6. A urea clearance above normal may be found in pregnancy, nephrosis, fevers, and after a high protein intake.

VI. Uric Acid (Modified Koch's Method).

A. ***Principle:*** Uric acid reduces the phosphotungstate reagent to a blue phosphotungstate compound. Substances in the blood other than uric acid, such as ergothioneine and glutathione, give this blue color, but these are eliminated to a large extent by using serum for the test.

B. *General Considerations.*

1. The serum is deproteinized with sulfuric acid and sodium tungstate (see directions on page 265) and allowed to stand 20 minutes before filtering.
2. The urea cyanide solution should be kept in the refrigerator at all times.
3. The urea cyanide solution is *poisonous* and should be handled with caution.

4. *Sources of error.*
    a. Urea cyanide solution not cold when added to the filtrate or standard.
    b. Using a dilute standard solution that has stood longer than 1 month.

C. *Method.*

1. Place 5 cc. of the protein-free serum filtrate in a large test tube and 5 cc of standard uric acid solution (5 cc. = 0.02 mg.) in another test tube of the same size.
2. Add 5 cc. of cold urea cyanide solution from a long tipped burette directly into the solution in each tube.
3. Mix by whirling at an angle of 60°.
4. Add 1 cc. of uric acid reagent dropwise directly into the solution in each tube.
5. Mix the contents of both tubes well and allow to stand 20 minutes in a 25°C. water bath before reading.
6. Read the unknown against the standard set at 20 mm. in a colorimeter.
7. *Calculation:*

$$\frac{RS}{RU} \times 0.02 \times \frac{100}{0.5} = \frac{80}{RU} = \text{mg. \%.}$$

D. *Solutions.*

1. *Urea Cyanide Solution.*
    a. Dissolve 250 gm. of Merck's or Baker's c.p. urea in 700 cc. of water.
    b. Filter into a liter volumetric flask and dilute to volume with water.
    c. This 25% solution is stable when stored in the refrigerator.
    d. Dissolve 2.5 gm. of Merck's or Baker's c.p. sodium cyanide in 100 cc. of the 25% urea solution. This is stable for 2 or 3 days when it is stored in the refrigerator.
    e. Check each new bottle of sodium cyanide by using a standard and an unknown filtrate for both old and new sodium cyanide reagents.
2. *Uric Acid Reagent.*
    a. Dissolve 100 gm. of sodium tungstate (Pfanstiehl, c.p.) in 700 cc. of water in a 2 liter Florence flask.
    b. Add 75 cc. of 85% phosphoric acid and boil gently under a reflux condenser for 24 hours. Place tin foil around the stopper connecting the condenser to the flask.
    c. Decolorize by adding a few drops of bromine or 30% hydrogen peroxide to the hot solution and boil without the condenser for 10 minutes to remove the excess.
    d. Transfer the pale yellow liquid to a liter volumetric flask and dilute to volume with water. Filter and store in a glass-stoppered brown bottle.
    e. Check each new reagent by using a standard and an unknown filtrate for both old and new reagents.
3. *Stock Standard Uric Acid Solution* (1 cc. = 1 mg.).
    a. Weigh out on a watch glass exactly 1 gm. of uric acid and transfer it to a liter volumetric flask by means of a dry funnel.
    b. Transfer 0.6 mg. of lithium carbonate to a 200 cc. Florence flask, add 150 cc. of water and shake until dissolved, about 5 minutes.
    c. Filter the carbonate solution and heat the filtrate to 60°C.
    d. Warm the liter flask containing the uric acid under running warm water and pour the warm lithium carbonate solution into it, washing into the flask the traces of uric acid which have adhered to the watch glass and funnel.
    e. Shake in order to dissolve the uric acid immediately. A little additional heating under running water is permissible.
    f. The lithium carbonate solution is not always perfectly clear even when filtered. This turbidity should not be mistaken for undissolved uric acid and the warming and shaking continued too long.
    g. In 5 minutes the uric acid should all be dissolved. Shake the flask under cool running water without undue delay.
    h. Add 20 cc. of neutral c.p. formalin (40% formaldehyde) and half fill the flask with water.
    i. Add a few drops of methyl orange indicator and finally add from a pipette, slowly and with shaking, about 25 cc. of N sulfuric acid.
    j. The solution should turn pink while 2 or 3 cc. of acid still remain in the pipette, showing that the total acidity from adding 25 cc. of acid is not too great.
    k. Dilute to volume with water, mix thoroughly, and transfer to a clean tightly stoppered bottle.
    l. Stored away from light, it will keep for at least 5 years.
4. *Dilute Standard Uric Acid Solution* (5 cc. = 0.02 mg.).
    a. Pipette 1 cc. of the stock standard uric acid solution into a 250 cc. volumetric flask and dilute to volume with water.
    b. Add 3 cc. of chloroform.
    c. Prepare fresh once a month.

terpretation of Blood Uric Acid Find- gs.

ormal Values: 2.0—5.0 mg. per cent.

creased in:

Renal insufficiency.

Acute and chronic nephritis
Urinary obstruction
Metallic poisoning, e.g. mercury and lead.
Hypertension, especially when uncompensated.

Metabolic disturbances.

Gout
Diabetes with acidosis
Following a high purine diet
After exercise

Conditions accompanied by massive and rapid destruction of cell nuclei.

Leukemia
Polycythemia
Pernicious or any severe anemia during stage of rapid regeneration of erythrocytes.
Malignant tumors especially with extensive necrosis.

Miscellaneous.

Acute infections
Cardiac decompensation
Intestinal obstruction
Eclampsia

ecreased in:

Pernicious anemia—relapse
Acute yellow atrophy of the liver
Salicylate and atophan therapy

VII. Creatinine (Folin's Method).

rinciple: The determination is based upon e Jaffé reaction between creatinine and dium picrate in alkaline solution to form a d tautomer of creatinine picrate. This rection is not specific for creatinine but also ccurs with other chromogenic substances hich may be ruled out in part by using serum filtrate instead of a whole blood ltrate.

eneral Considerations.

ince high creatinine values are always acompanied by increases in urea and uric acid, is only necessary to run the creatinine etermination when these are high.

he unknown *must* closely approximate the olor of the standard with which it is comared in the colorimetric method.

the value for urea nitrogen is available, t up a creatinine standard equal to the urea itrogen value divided by ten. This will sually give a close approximation of color standard and unknown.

4. *Sources of error.*
   a. Using alkaline picrate that has not stood 20 minutes nor longer than 1 hour.
   b. Not thorough mixing of solution after adding alkaline picrate solution.
   c. Incorrect timing before reading in the photoelectric colorimeter.

C. *Colorimetric Method.*

1. Place 10 cc. of protein-free serum filtrate in a large test tube.
2. Set up two standards.
   a. Ten cc. of the creatinine standard solution (10 cc. = 0.03 mg.) in one test tube.
   b. Five cc. of the creatinine standard solution (5 cc. = 0.015 mg.) plus 5 cc. of water in another test tube.
3. Add 5 cc. of the alkaline picrate solution (made fresh) to each of the 3 test tubes and mix.
4. Set the standard that most closely approximates the color of the unknown in the colorimeter at 20 mm.
5. Read the unknown 8 to 10 minutes after adding the alkaline picrate solution.
6. The color comparisons must be completed at the end of 15 minutes; therefore, it is never advisable to work with more than 3 to 5 unknowns at a time.
7. If the creatinine content of the unknown is too high for the strongest standard, it may be diluted with 10 cc. of water and 5 cc. of alkaline picrate and then read in the colorimeter, but always within the time limits set above.
8. *Calculation:*

$$\frac{\text{RS}}{\text{RU}} \times 0.015 \times \frac{100}{1} = \frac{30}{\text{RU}} = \text{mg. \%.}$$

$$\frac{\text{RS}}{\text{RU}} \times 0.03 \times \frac{100}{1} = \frac{60}{\text{RU}} = \text{mg. \%.}$$

D. *Photoelectric Colorimeter Method.*

1. Pipette 10 cc. of water into a colorimeter tube for a blank.
2. Pipette 10 cc. of protein-free serum filtrate into another colorimeter tube.
3. Place the 2 colorimeter tubes in a 25°C. water bath and allow 5 minutes for them to assume the temperature of the water bath.
4. Blow 5 cc. of freshly made alkaline picrate solution into each tube while shaking it, and let the tubes stand in the water bath for exactly 10 minutes.
5. Set the galvanometer at 100 using the blank and filter No. 520.
6. Read the unknown and obtain the mg. per cent of creatinine from the table of values.

7. *Calibration of Standard Curve.*
   a. Make a dilute standard solution by placing 10 cc. of the stock standard creatinine solution in a liter volumetric flask and diluting to volume with 0.1 N HCl (1 cc. = 0.01 mg.).
   b. Pipette the following amounts of the dilute standard solution and water into a series of colorimeter tubes that have been placed in a water bath at 25°C.

cc. of dilute standard solution	cc. of water	mg. of. creatinine in 10 cc.	mg. % of creatinine in blood
1	9	0.01	1
2	8	0.02	2
3	7	0.03	3
4	6	0.04	4
5	5	0.05	5
6	4	0.06	6
7	3	0.07	7
8	2	0.08	8
9	1	0.09	9
10	0	0.10	10
0	10	Blank	Blank

   c. Continue as described in the method above.
   d. Repeat tests several times using the same dilute standard solution and then repeat on several new dilute standard solutions.
   e. Average the galvanometer readings for each concentration and plot on semilogarithmic graph paper. It should make a straight line.
   f. Make a table of values according to mg. per cent of creatinine in blood for each galvanometer division.

E. Solutions.

1. *Saturated Picric Acid Solution*—1.17%.
   a. Dissolve 13.5 gm. of purified picric acid in 900 cc. of water with the aid of heat.
   b. Cool and store in a brown bottle.
   c. *Purification of picric acid.*
      1) Dissolve 125 gm. of anhydrous sodium carbonate and 250 gm. of picric acid in 3000 cc. of boiling water.
      2) Filter while hot and set the clear filtrate aside at room temperature for 12 to 24 hours.
      3) Collect the crystallized sodium picrate on a Buchner funnel, pack firmly with a pestle, and wash by suction on the funnel with about 1000 cc. of cold 10% NaCl solution.
      4) Avoid the development of cracks in the cake of sodium picrate on the filter.
      5) Suspend the caked precipitate in about 150 cc. of cold water and acidify by the gradual addition of conc. HCl (about 80 cc.) until the orange sodium picrate changes completely to the yellow color of picric acid. The sodium picrate is changed by the HCl to picric acid which crystallizes out.
      6) Filter through a Buchner filter.
      7) Suspend the precipitate in ice cold water and refilter. Wash in this way at least 3 times.
      8) Dry the picric acid thus obtained between filter papers or in a large evaporating dish and store in a dark bottle.
   d. *Test for purity of picric acid.*
      1) Prepare a saturated solution of picric acid.
      2) Add 0.5 cc. of 10% NaOH to 10 cc. of the saturated solution of picric acid and let stand for 15 minutes.
      3) Compare this alkaline solution in a colorimeter with the saturated picric acid solution set at 20 mm.
      4) The alkaline solution, when set to match the saturation solution, should not read less than 13.5 mm.
2. *Alkaline Picrate Solution.*
   a. To 25 cc. of the saturated picric acid in a 125 cc. Erlenmeyer flask, add 5 cc. of 10% NaOH and mix well.
   b. This solution must be made daily and allowed to stand 20 minutes before adding to the filtrate, but it can not be used after standing longer than 1 hour.
3. *Standard Creatinine Solutions.*
   a. *Stock Standard Solution* (1 cc. = 1 mg.).
      1) Dissolve 100 mg. of creatinine in about 80 cc. of 0.1 N HCl in a 100 cc. volumetric flask.
      2) Dilute to volume with 0.1 N HCl.
   b. *Dilute Standard Solution* (10 cc. = 0.03 mg.).
      1) Place 3 cc. of the stock solution in a liter volumetric flask, add 10 cc. of 0.1 N HCl, and dilute to volume with water.
      2) Add a few drops of chloroform or toluene.

F. *Interpretation of Blood Creatinine Findings.*

1. *Normal Values:* 0.8—2 mg. per cent.
2. *Increased in:*
   Early nephritis (2—4 mg.)
   Severe nephritis (4—35 mg.)
   Urinary obstruction
   Intestinal obstruction
3. *Decreased in:*
   Amyotonia congenita

III. Urine Creatinine and Creatine.

inciple: The creatine in the urine is con-
ted to creatinine by boiling with hydro-
oric acid and granulated lead. The total
eatinine in the boiled specimen minus the
eatinine in the untreated urine gives the
eatine value.

eatine Tolerance Test.

ring the 3 days of the test, the patient is
pt in bed, placed on a creatine-free diet
o meat, fish, fowl, soups, cocoa, or choco-
e), and given 1500 cc. of fluid each 24
urs.

ch 24 hour urine specimen is collected in
bottle containing 10 cc. of toluene and
pt in the refrigerator.

ne control urine (first 24 hour specimen)
collected and then 1.0 gm. of creatine is
ven orally in one-half glass of water.

nen 24 hour urine specimens are collected
r 2 days.

ormally most of the ingested creatine is
anged to creatinine, increasing the output
creatinine in the next two 24 hour speci-
ens.

muscular dystrophy the creatine is not
anged to creatinine as rapidly as in a nor-
al person; therefore, there is a greater in-
ease of creatine in the next two 24 hour
ine specimens than in normal individuals.

olorimetric Method.

ix the 24 hour urine specimen and measure
e volume.

otal Creatinine.

Transfer 1 cc. and 2 cc. of urine to
medium sized pyrex test tubes.

Add 2 cc. of approximately normal HCl
(10%) and a very small amount of gran-
ulated lead to each.

Boil and evaporate the contents of the
tubes to 0.5 cc. volume over an open
flame.

. Add 2 cc. of hot water and allow to
stand for a few minutes.

. Transfer each quantitatively to 100 cc.
volumetric flasks through a small cotton
plug in a small funnel.

. Wash the tubes with a 2 cc. portion of
hot water and then with 1 cc. of hot
water and transfer all washings to the
volumetric flasks.

Preformed Creatinine.

. If urine is alkaline, acidify a few cc. with
acetic acid until a pH of 6.4 is attained
using nitrazine paper.

. Place 1 cc. and 2 cc. of urine in 100 cc.
volumetric flasks.

c. Add water to bring the volume of each to 5 cc.

4. *Standard*—place 1 cc. of the stock creatinine standard solution (1 cc. = 1.0 mg.) in a 100 cc. volumetric flask.
5. Add 20 cc. of a saturated picric acid solution to each of the 5 flasks.
6. Add 2.5 cc. of the Rochelle salt reagent to each flask.
7. Allow to stand 10 minutes, then dilute to volume with water and read the dilution of each determination that is the closest match to the standard which is set at 20 mm.
8. *Calculation:*

$$\frac{RS}{RU} \times 1.0 \times \frac{\text{vol. of urine}}{\text{vol. used}} = \text{mg. per 24 hours.}$$

Creatine expressed as creatinine = total creatinine — preformed creatinine.

D. *Photoelectric Colorimeter Method.*

1. Mix the 24 hour urine specimen and measure the volume.
2. *Total Creatinine.*
   a. Place 2 cc. of urine in a medium sized pyrex test tube.
   b. Add 2 cc. of approximately normal HCl (10%) and a very small amount of granulated lead.
   c. Boil and evaporate the contents of the tube to approximately 0.5 cc. volume over an open flame.
   d. Add 2 cc. of hot water and allow to stand for a few minutes.
   e. Transfer quantitatively to a 100 cc. volumetric flask through a small cotton plug in a small funnel, washing the tube and cotton plug thoroughly with hot water.
   f. Dilute to volume with water and mix. This makes a 1-50 dilution.
   g. Make a 1-250 dilution by placing 10 cc. of the 1-50 dilution in a 50 cc. volumetric flask, diluting to volume with water and mixing.
3. *Preformed Creatinine.*
   a. If urine is alkaline, acidify about 10 cc. with acetic acid until a pH of 6.4 is attained with nitrazine paper.
   b. Place 5 cc. of urine in a 100 cc. volumetric flask, dilute to volume with water, and mix. This makes a 1-20 dilution.
   c. Make a 1-200 dilution by placing 5 cc. of the 1-20 dilution in a 50 cc. volumetric flask, diluting to volume with water, and mixing.
4. Transfer 10 cc. of the 1-250 dilution for total creatinine into one colorimeter tube.

5. Transfer 10 cc. of the 1-200 dilution for preformed creatinine into another colorimeter tube.
6. Pipette 10 cc. of water into another colorimeter tube for the blank.
7. Place the 3 colorimeter tubes in a 25°C. water bath and allow 5 minutes for them to assume the temperature of the water bath.
8. Blow 5 cc. of freshly made alkaline picrate solution (see solutions for blood creatinine) into each tube while shaking it and let the tubes stand in the water bath for exactly 10 minutes.
9. Set the galvanometer at 100 using the blank and filter No. 520 and then read the unknowns.
10. *Calculation:*
    - a. For the 1-200 dilution divide the mg. per cent of creatinine in the table of values for blood by 100 and multiply by 20 to obtain the mg. of creatinine in 1 cc. of urine. For mg. of creatinine in the 24 hour specimen, multiply by the number of cc. in the specimen.
    - b. For the 1-250 dilution multiply by 25 instead of 20 in the above calculation.
    - c. Creatine expressed as creatinine = total creatinine—preformed creatinine.
11. If the creatine and creatinine are high, add 5 cc. of water to 5 cc of the urine dilutions in a colorimeter tube and then proceed with adding the picrate solution. Correct the final results for the extra dilution.
12. If the creatine and creatinine are low, lesser dilutions must be made from the first dilutions of 1-20 for preformed creatinine and 1-50 for total creatinine.

E. *Solutions.*

1. *Rochelle Salt Reagent.*
    - a. Place 5 gm. of Rochelle salts ($NaKC_4H_4O_6 \cdot 4H_2O$) in a 100 cc. volumetric flask and add about 60 cc. of 10% NaOH.
    - b. When the Rochelle salts are dissolved, dilute to volume with 10% NaOH.
2. *Stock Creatinine Standard Solution*—same as for blood creatinine (p. 286).

F. *Interpretation of Urine Creatine and Creatinine.*

1. *Creatine.*
    - a. *Normal Values.*
      Men: 0—50 mg. per 24 hours.
      Women: 0—100 mg. per 24 hours.
    - b. *Increased in:*
      Muscular disorders such as:
        - Myasthenia gravis
        - Muscular atrophy
        - Pseudohypertrophic muscular dystrophy
        - Amyotonia congenita
        - Myotonia atrophica
        - Anterior poliomyelitis
        - Congenital muscular hypertrophy
        - Amyotrophic lateral sclerosis
        - Myostitis fibrosa
        - Diffuse myostitis

      Hepatic carcinoma
      Hyperthyroidism
      Phosphorus poisoning
      Diabetes
      Starvation
      Convulsions
      Carbon monoxide poisoning
      High water intake
      Fevers
2. *Creatinine.*
    - a. *Normal Values:* 0.7—2.0 gm. per 24 hours. (Range of 25 to 32 mg. per kg. of body weight.)
    - b. *Increased in:*
      Diabetes
      Typhoid fever
      Pneumonia
      Tetany
    - c. *Decreased in:*
      Muscular atrophy
      Anemia
      Advanced nephritis
      Leukemia
3. *Creatine Tolerance Test.*
    - a. After the ingestion of 1 gm. of creatine, a normal individual may excrete up to 300 mg. of creatine in 24 hours.
    - b. In muscular dystrophy the excretion is greater than 300 mg., usually about 600 mg. of creatine.

Lipids

I. Total Serum Cholesterol (Bloor's Method).

A. *Principle:* The lipoid is extracted from serum with a hot alcohol-ether mixture. The extract is evaporated to dryness and the cholesterol is extracted from the residue with chloroform. The Liebermann-Burchard color reaction is developed with acetic anhydride and concentrated sulfuric acid and either compared in the colorimeter with that of a standard or read in a photoelectric colorimeter.

B. *General Considerations.*

1. No more than 4 bloods (8 readings) should be read with one standard in the colorimeter.
2. *Sources of error.*
    - a. All apparatus and pipettes must be absolutely dry as any moisture will interfere with the color development.

Scorching of the residue while evaporating
the alcohol-ether filtrate.

Careless pipetting of the chloroform ex-
tract; chloroform is difficult to pipette
because the surface tension is less than
that of an aqueous solution.

Careless pipetting of sulfuric acid.

Timing of the color development not
exact.

Incorrect temperature of water bath.

Evaporation of filtrates before pipetting.

olorimetric Method.

dd 1 cc. of serum dropwise to approxi-
ately 40 cc. of the alcohol-ether mixture
a glass-stoppered 50 cc. volumetric flask
ith constant shaking in order to avoid
lumping of the protein.

Ieat to boiling in a hot water bath, ro-
ating the flask while heating to avoid loss
f solution by boiling over.

ool the solution to room temperature,
ilute to volume with the alcohol-ether mix-
ure, and mix.

lace all of the solution in a fat-free filter
aper and allow all of it to filter into a 60
c. glass-stoppered bottle. Stopper immedi-
tely and mix.

lace 10 cc. of the filtrate in each of two
0 cc. beakers and evaporate just to dry-
ess over a steam or sand bath.

Add 2 cc. of chloroform to each beaker
while it is still warm and allow to stand
until cooled.

Transfer the chloroform extract quantita-
tively to a glass-stoppered 10 cc. cylinder.

Wash the beaker twice with 2 cc. portions
of chloroform and transfer each quanti-
tatively to the cylinder. Make the extract
up to 6 cc. with chloroform.

Standard.

a. Place 5 cc. of the cholesterol standard solution (5 cc. = 0.5 mg.) measured at 20°C. in a 10 cc. cylinder.
b. Make up to 6 cc. with chloroform at room temperature.

Place the unknowns and standard in a 25°C. water bath and add 2 cc. of acetic anhydride and exactly 0.1 cc. of conc. sulfuric acid to each.

Stopper and mix by inverting.

Allow the color to develop in the dark for exactly 25 minutes.

Read in the colorimeter against the standard set at 20 mm. Dry cups and prisms completely before using them.

Calculation:

$$\frac{RS}{RU} \times 0.5 \times \frac{100}{0.2} = \frac{5000}{RU} = \text{mg. \%.}$$

D. ***Photoelectric Colorimeter Method.***

1. Prepare a 1-50 alcohol-ether extract of serum (steps 1 through 4 in colorimetric method).
2. Place 20 cc. of the filtrate in a 100 cc. beaker and evaporate just to dryness over a steam bath.
3. Add 8 cc. of chloroform to the beaker while it is still warm and allow to stand until cooled.
4. Transfer quantitatively to a glass-stoppered 25 cc. volumetric flask.
5. Wash the beaker twice with 8 cc. portions of chloroform and transfer each quantitatively to the flask.
6. Dilute to volume with chloroform at room temperature, stopper, and mix the contents by repeatedly inverting the flask. (Do not dilute to volume until ready to pipette aliquot portions in the next step.)
7. Place 10 cc. of the extract in each of 2 colorimeter tubes, draining the pipette slowly.
8. Prepare a blank by placing 10 cc. of chloroform in another colorimeter tube.
9. Place all the tubes in a 25°C. water bath.
10. Add 2 cc. of acetic anhydride to each tube and mix by whirling the tube.
11. Add exactly 0.1 cc. of conc. sulfuric acid slowly to each tube, placing the tip of the pipette just above the surface of the solution and allowing the acid to run down the side of the tube.
12. Mix by whirling the tube.
13. Allow the color to develop in the dark for exactly 25 minutes.
14. Place the tube containing the blank in the colorimeter using filter No. 660. Adjust the galvanometer to 100, remove the tube, and take the galvanometer reading.
15. With the galvanometer set at this reading, obtain the reading of each unknown. The duplicates of each unknown must check.
16. The cholesterol value is obtained from the table of values.
17. *Calibration of Standard Curve.*
    a. Prepare the following standard solutions by carefully weighing the listed amounts of cholesterol, placing the first 5 in liter volumetric flasks and the last 4 in 500 cc. volumetric flasks.
    b. Dilute to volume with chloroform at 20°C.
    c. The standards are weighed instead of pipetted from a stock solution because chloroform is difficult to pipette accurately.

mg. of cholesterol	mg. in 10 cc.	mg. % in serum
10	0.1	62.5
20	0.2	125.0
30	0.3	187.5
40	0.4	250.0
50	0.5	312.5
30	0.6	375.0
40	0.8	500.0
50	1.0	625.0
60	1.2	750.0

d. Develop the color in 10 cc. portions of each standard as described above (7—15).

e. Perform a sufficient number of determinations on each concentration of cholesterol so that an average of the readings obtained will give a straight line when plotted on semilogarithmic graph paper.

f. Make a table of values for each galvanometer division.

E. *Solutions.*

1. *Alcohol-Ether Mixture*—three parts of absolute ethyl alcohol and 1 part of anhydrous ether.
2. *Fat-free filter paper* can be made by soaking the paper in ether and drying.
3. *Chloroform*—reagent grade.
4. *Acetic Anhydride*—must be pure and colorless.
5. *Standard Cholesterol Solution.*
    a. *Stock standard solution* (1 cc. = 1 mg.).
        1) Place 200 mg. of cholesterol (m. p. 148°C.) in a 200 cc. volumetric flask.
        2) Make up to volume at 20°C. with chloroform.
    b. *Dilute standard solution* (5 cc. = 0.5 mg.).
        1) Pipette 10 cc. of stock standard solution into a 100 cc. volumetric flask.
        2) Dilute to volume with chloroform at 20°C.
        3) Great care should be taken in making this standard solution because of the difficulty in pipetting chloroform. It should be checked with another standard solution.

F. *Interpretation of Serum Cholesterol Findings.*

1. *Normal Values.*
   Women: 180—260 mg. per cent.
   Men: 150—250 mg. per cent.
2. *Increased in:*
   Diabetes mellitus
   Hypothyroidism
   Nephrosis
   Chronic glomerulonephritis
   Obstructive jaundice
   Xanthomatosis
   Celiac disease
   Lipemia
   Multiple sclerosis
   Leukemia
   Hemophilia
   Pregnancy
   Eclampsia
3. *Decreased in:*
   Anemia
   Hepatic insufficiency
   Infections—acute
   Hyperthyroidism
   Gaucher's disease
   Polycythemia
   Inanition
   Intestinal obstruction
   Epilepsy

II. Serum Cholesterol Esters (Bloor and Knudson's Method).

A. *Principle:* A one per cent solution of digitonin added to the alcohol-ether extract of serum combines with the free cholesterol to form cholesterol-digitonide. The filtrate is evaporated to dryness and the residue is extracted with petroleum ether. The cholesterol ester is dissolved by the petroleum ether separating it from the insoluble cholesterol-digitonide. The petroleum ether extract is evaporated to dryness and the ester is extracted with chloroform and is determined in the same manner as the total cholesterol.

B. *General Considerations.*

1. Same as for total cholesterol.
2. Test must be done within 1 hour after the blood is obtained from the patient because the esters are converted to free cholesterol on standing.

C. *Colorimetric Method.*

1. Prepare an alcohol-ether extract of serum using 2 cc. of serum in a 100 cc. volumetric flask and filter as described under total cholesterol.
2. Place 10 cc. of the filtrate in each of two 50 cc. beakers and proceed as described under total cholesterol.
3. For the esters place 20 cc. of the filtrate in a 100 cc. beaker and add 1 cc. of 1% digitonin (do in triplicate).
4. Evaporate just to dryness over a steam or sand bath.
5. Add 20 cc. of petroleum ether and evaporate on a sand bath to a volume of about 10 cc.
6. Transfer this quantitatively through a fat-free cotton plug in a small funnel into a 50 cc. beaker.
7. Add 20 cc. of petroleum ether to the 100 cc. beaker and again evaporate to 10 cc.

ransfer through the cotton plug into the rst extract. Repeat this process once more.

vaporate the combined petroleum extracts ıst to dryness.

dd 2 cc. of chloroform to the residue ı the beaker and continue as in total chosterol.

alculation:

$$\frac{RS}{RU} \times 0.5 \times \frac{100}{0.4} = \frac{2500}{RU} = \text{mg. \%.}$$

hotoelectric Colorimeter Method.

repare an alcohol-ether extract of serum s described under total cholesterol (colornetric method) using 2 cc. of serum in a 00 cc. volumetric flask.

lace 20 cc. of the filtrate in each of three 00 cc. beakers marked A, B, and C.

roceed with "A" as for total cholesterol.

o "B" and "C" add 1 cc. of 1% digitonin.

vaporate just to dryness over a steam bath.

o each beaker add 20 cc. of petroleum ther and evaporate on a sand bath to a olume of about 10 cc.

ransfer each quantitatively through fatree cotton plugs in small funnels to 50 cc. eakers marked "B" and "C."

Add 20 cc. of petroleum ether to each of the 100 cc. beakers and again evaporate to bout 10 cc.

Transfer each of these washings through he same cotton plugs into the 50 cc. beakers containing the first washing.

Repeat the process of washing and evaporating the contents of the 100 cc. beakers with a third portion of petroleum ether.

Evaporate each of the combined petroleum ether washings of "B" and "C" just to dryness.

Add 8 cc. of chloroform to each and proceed as described under total cholesterol (photoelectric colorimeter method 3—16).

olutions.

igitonin—1%.

. Place 1 gm. of digitonin in a 100 cc. volumetric flask and dissolve in about 50 cc. of 45% alcohol by heating in a warm water bath.

. Dilute to volume with 45% alcohol.

at-free Cotton—extract soft cotton 3 times with petroleum ether.

nterpretation of Serum Cholesterol Ester indings.

ormal Values: 70—75% of total cholesterol.

ncreased in: Lipoid nephrosis, amyloid nephrosis, chronic glomerulonephritis, and xanthomatosis.

ecreased in: Degenerative liver disease.

III. Lipids in Feces (Fowweather's Method).

A. ***Principle:*** Total fat includes free fatty acids, soaps, and neutral fat fractions. Hydrochloric acid is added to a portion of feces to convert the soaps to fatty acids. Then the total fat is removed in an ether extraction, purified by means of petroleum ether, and determined gravimetrically. Another portion of feces is not treated with HCl but is immediately extracted with ether and the amount of free fatty acids and neutral fats determined gravimetrically. From this precipitate the free fatty acids are dissolved in benzene and titrated with 0.1 N sodium alcoholate.

B. *General Considerations.*

1. The analysis should be made on fresh feces as fat decreases on standing even in the frozen condition.
2. Blank determinations should be made to rule out any fatty substance in the reagents.
3. The amount of dry matter—and likewise the water content—is extremely variable in the normal stool. Values should be expressed in terms of dry matter to be significant.
4. Drying alters the chemical composition of feces so the lipoids are determined in "wet" feces but reported as per cent of "dry" feces.

C. *Method.*

1. Weigh 3 stoppered weighing bottles labeled A, B, and C on an analytical balance.
2. Mix the total stool specimen thoroughly in a mortar with the aid of a pestle.
3. Place about 2 or 3 gm. of feces (5 gm. if stool is liquid) in each of the weighed bottles and weigh accurately.
4. Place bottle C on a steam bath or in a drying oven (95 to 100°C.) until the fecal matter is dried to a constant weight; this gives the per cent of dry matter present.
5. With the aid of a stirring rod, transfer the contents of bottle A to a 100 cc. glass-stoppered cylinder labeled A.
6. Care must be taken not to contaminate the neck of the cylinder with feces.
7. Add 3 cc. of conc. hydrochloric acid to the cylinder and then use small portions of water to rinse the contents of the weighing bottle into the cylinder, making the contents up to 30 cc. (Cylinder A for total fat.)
8. Transfer the contents of Bottle B to a 100 cc. glass-stoppered cylinder labeled B and use small portions of water to rinse the

contents of the weighing bottle into the cylinder making the contents up to 30 cc. (Cylinder B for fatty acids plus neutral fat.)

9. Add 20 cc. of anhydrous ether to each cylinder and shake vigorously for 5 minutes.
10. Let stand a few minutes and then add 17 cc. of 95% alcohol to Cylinder A and 20 cc. to Cylinder B.
11. Mix the contents of the cylinders by a quick rotary motion and cool to room temperature in running water.
12. Shake the contents vigorously for 5 minutes and allow to stand until the ether layer separates. Separation may be expedited by occasional rotation of the cylinder or by adding a few drops of alcohol or ether.
13. The ether layer is blown off as completely as possible into a 200 cc. Erlenmeyer flask in the same manner that water is blown from a wash bottle. The submerged end of the delivery tube is bent upward, this avoids upward currents which would disturb the subjacent alcohol-ether-feces layer.
14. Another 20 cc. of ether is then added, the cylinder shaken for 5 minutes, and the ether allowed to separate.
15. Blow off the ether layer into the same flask and repeat the ether extraction again.
16. Wash the stopper and sides of the cylinder with 3 successive 5 cc. portions of ether and add each to the previous ether extractions.
17. Evaporate the combined extract and washings to dryness.
18. Add 20 cc. of petroleum ether, warm on a water bath, and filter through fat-free filter paper into a tall previously weighed 100 cc. beaker.
19. Repeat twice using 10 cc. portions of petroleum ether.
20. Evaporate the petroleum ether, then dry the residue to a constant weight in a 37°C. oven, cool to room temperature, and weigh.
21. Weight of residue in beaker containing extract of Cylinder A is the total fat (A).
22. Weight of residue in beaker containing extract of Cylinder B is the free fatty acids plus neutral fat (B).
23. Dissolve the residue from Cylinder B in about 50 cc. of benzene and heat almost to boiling.
24. Titrate while still hot wtih 0.1 N sodium alcoholate, using 2 drops of phenolphthalein as an indicator.
25. Titrate until the color no longer deepens.

D. *Calculation:*

1. *Dry Matter.*

$$\frac{\text{Dried weight (C)}}{\text{Net weight (C)}} \times 100 = \text{per cent of dry matter}$$

2. *Total Fat.*
   a. Calculate the weight of dry matter of specimen A by multiplying the wet weight by the per cent of dry matter found in specimen C.
   b. Calculate gm. of fat per gm. of dry matter of specimen A.
   c. To express in per cent multiply by 100.
3. *Free Fatty Acid plus Neutral Fat.*
   a. Calculate the weight of dry matter of specimen B by multiplying the wet weight by the per cent of dry matter found in specimen C.
   b. Calculate gm. of free fatty acids plus neutral fat per gm. of dry matter of specimen B.
   c. To express in per cent multiply by 100.
4. *Free Fatty Acids.*
   a. One cc. of 0.1 N sodium alcoholate titrates 28.2 mg. of oleic acid or 28.4 mg. of stearic acid (average 28.3).
   b. Number of cc. of 0.1 N sodium alcoholate used in the titration × 28.3 = mg. of free fatty acid in specimen B.
   c. Calculate gm. of free fatty acids per gm. of dry matter of specimen B.
   d. To express in per cent multiply by 100.
5. *Soaps as Fatty Acids.*
   (Total fat) — (free fatty acids plus neutral fat) = soaps in terms of fatty acids.
6. *Neutral Fat.*
   (Free fatty acid plus neutral fat) — (free fatty acids) = neutral fat.

E. *Solutions.*

1. *Sodium Alcoholate*—0.1 N.
   a. Place about 500 cc. of absolute ethyl alcohol (redistilled) in a liter volumetric flask.
   b. Add 2.3 gm. of freshly cut metallic sodium, when dissolved dilute to volume with alcohol.
   c. Titrate with 0.1 N HCl using 2 drops of 0.5% alcoholic phenolphthalein as an indicator.
   d. Adjust solution to 0.1 N if necessary.
   e. Keep solution in refrigerator and discard when it becomes colored.
2. *Petroleum Ether*—boiling point should be below 60°C.
3. *Benzene* (C_6H_6).

erpretation of Fecal Lipid Findings.

ormal Values:

y matter: 4.6—38%.

otal fat: 7.3—27.6 gm.%.

ee fatty acids: 1.05—10 gm.%.

aps as fatty acids: 0.54—11.4 gm.%.

eutral fat: 2.49—11.8 gm.%.

eutral fat as per cent of total fat: 24.6—.1%.

normal Values:

Celiac disease and obstructive jaundice—increase in total fat, soaps, and free fatty acids; neutral fat is normal.

Pancreatic deficiency—increase in total fat, neutral fats, soaps, with normal or low fatty acids.

Nontropical sprue—increase in total fat, fatty acids, and neutral fats.

Tropical sprue—increase in total fat and fatty acids, and decrease in neutral fat.

Gastro-enteritis—increase in all lipid fractions.

Gas Analysis

I. Acid-Base Balance.

arbon dioxide is constantly formed in the ody and promptly unites with water to orm H_2CO_3. This in turn binds all bases ot bound by other acids to form bicarbo-ate; the excess H_2CO_3 is given off by the ings as CO_2. The bicarbonate formed rep-esents the excess base which is left after l the nonvolatile acids have been neutral-ed and is called the alkaline reserve of the ody. *Alkalosis* is an increase in the alkali eserve which may or may not be associated ith a rise in blood pH.

cids invade the blood in both normal and athologic metabolism and bind some of he alkali. Normally the kidneys eliminate hese acids and retain the alkali; in this way he kidneys excrete an acid urine (pH 5 to) from an alkaline blood (pH 7.4). *Acido-is* is a condition caused by acid retention ufficient either to lower the reserve bi-arbonate below normal or to shift the pH of the blood toward the acid side. Diabetic cidosis is due to the abnormal formation of diacetic and the beta-hydroxybutyric cids; acidosis in nephritis is due to the failure of the kidneys to eliminate phosphoric and sulfuric acids. In both cases the available alkali is bound by these acids and excreted, thus reducing the alkaline reserve of the body. The pH of the blood remains normal in acidosis unless the respiratory apparatus is affected to the extent that the excess CO_2 cannot be eliminated.

II. Determination of the pH of the Serum (Cullen's Method).

A. ***Principle:*** Certain substances called indicators change color when the hydrogen ion concentration as expressed in pH is altered. When an indicator is added to a diluted sample of serum, the resulting color may be determined colorimetrically.

B. ***General Considerations.***

1. There must be no hemolysis of the blood.
2. The 20 cc. syringe must be rinsed with distilled water and dried before using.
3. The blood must be collected and centrifuged under all possible anaerobic conditions to prevent the loss of CO_2.
   a. Place enough mineral oil in a tube so that the blood can be expelled into the tube under the oil.
   b. Coat the inside of the 20 cc. syringe with mineral oil and obtain 10 cc. of venous blood without the use of a tourniquet.
   c. Place the blood in the tube under oil and when clotted loosen the clot with a stirring rod.
   d. Close the tube with a one-hole rubber stopper and then remove the oil remaining over the blood by inserting a fine capillary pipette through the hole in the stopper.
   e. Close the hole in the rubber stopper with a glass plug and centrifuge the tube.
   f. Cover the surface of the serum with oil by adding the oil through the hole in the stopper.
   g. The serum is transferred to another test tube containing oil and is kept under oil until the test is finished.
4. Water, mineral oil, phenol red, and glassware should be tested for neutrality.
   a. Water should give no red color when tested with both phenol red and methyl red.
   b. Oil is tested by shaking with water containing phenol red and methyl red.
5. Determination should be completed within 30 minutes after blood is centrifuged.

C. ***Photoelectric Colorimeter Method.***

1. Obtain serum as described under general considerations.
2. Pipette 10 cc. of freshly prepared 0.9% NaCl solution into each of two colorimeter tubes marked 1 and 2.
3. To tube 1 add 1 drop of 0.1% phenol red. Adjust the pH to approximately 7.4 (first faint pink) by adding a small drop or two of 0.02 N NaOH.

4. Cover saline with a layer of mineral oil and place tubes in a 20°C. water bath.
5. Fill a 1 cc. graduated pipette to the zero mark with serum by introducing the pipette through the layer of oil above the serum.
6. Quickly insert the tip of the pipette through the layer of oil covering the saline-phenol red mixture in tube 1.
7. Add 0.5 cc. of serum to tube 1 and stir the contents of the tube with the tip of the pipette before removing it.
8. Add the remaining 0.5 cc. of serum to the 10 cc. of saline in tube 2.
9. Read tube 1 after setting the galvanometer at 100 using tube 2 as the blank and filter No. 565.
10. Read tube 1 again after setting the galvanometer at 100 using tube 2 as the blank and filter No. 420.
11. Take the temperature of the content of tube 1 by immersing a thermometer in it.
12. *Calculation:*

$$R = \frac{\text{L value with filter 565}}{\text{L value with filter 420}}$$

R = ratio.

L = 2—log of the galvanometer reading.

   a. Obtain the pH for R from the table of values for R; interpolate if necessary.
   b. The true pH of the serum at 38°C. is determined by applying a correction factor.

True pH = uncorrected pH — [0.42 — (0.01 × t)].

t = temperature of the content of tube 1.

D. *Calculation of R Values.*

1. Prepare M/15 Na_2HPO_4 and M/15 KH_2PO_4 solutions as described under buffer solutions on page 259.
2. Prepare 100 cc. of 10 standards having pH values ranging from 7.0 to 8.0 as directed in Table 79, page 260.
3. Before using for curve, check the pH of each standard solution with a pH meter.
4. Place 10 cc. portions of these standard solutions in colorimeter tubes, add 1 drop of 0.1% phenol red, and read at 20°C. in the colorimeter with filters No. 565 and 420 using a blank consisting of one of the buffer solutions to set the galvanometer at 100 for each filter.
5. Repeat several times until consistent results are obtained.
6. Calculate the "R" values for each pH.
7. Make a table of values for "R" and the pH readings.
8. The following "R" values with their corresponding pH values may be used for an Evelyn instrument.

R	pH	R	pH
0.417	7.0	1.550	7.
0.521	7.1	1.910	7.
0.651	7.2	2.340	7.
0.812	7.3	2.850	7.
1.010	7.4	3.450	8.
1.260	7.5		

E. *Solutions.*

1. *Phenol Red Indicator* (pH range 6.8—8.4).
   a. Weigh exactly 100 mg. of phenol red and place in a 100 cc. volumetric flask.
   b. Add about 50 cc. of water and 28.2 cc. of 0.01 N NaOH and dilute to volume with water.
   c. Keep in a dropper bottle which delivers 25 drops to 1 cc.
   d. The indicator must be neutral.
      1) Test by adding 1 drop of indicator to 3 cc. of redistilled water.
      2) If neutral, the water will not turn red.
   e. Indicator color change is yellow to red in acid to alkaline solution.
2. *Sodium Chloride Solution*—0.9% freshly prepared.
3. *Mineral Oil*—neutral c.p.
4. *Sodium Hydroxide*—0.02 N.
5. *Sodium Hydroxide*—0.01 N.

F. *Interpretation of Serum pH Findings.*

1. *Normal pH of Serum:* 7.35—7.45.
2. *Decreased* in uncompensated acidosis.
3. *Increased* in uncompensated alkalosis.

III. Carbon Dioxide Combining Power (or Capacity) of Serum (Van Slyke and Cullen's Method).

A. *Principle:* Serum is saturated with carbon dioxide and the latter is liberated from the serum in vacuum by adding an acid. The volume of the liberated gas is measured at room temperature and atmospheric pressure and is reported under standard conditions.

$$2\ NaHCO_3 + H_2SO_4 \rightarrow Na_2SO_4 + 2\ H_2O + 2\ CO_2 \uparrow.$$

B. *General Considerations.*

1. Do not use a tourniquet when drawing the blood; if it is required to locate the vein, release before withdrawing the blood.
2. There must not be any hemolysis of the blood because it will give a lower result.
3. The carbon dioxide combining power of serum must be determined within 30 minutes after the blood is drawn.
4. If plasma is used, the blood must be drawn under oil.
5. The patient should avoid muscular exertion for at least 1 hour before the blood is drawn, or the value will be lower than normal.

urces of error.

Apparatus not air tight.

Incomplete saturation of serum or plasma with CO_2.

Allowing air to enter the apparatus while doing the test.

ethod.

eparation of the CO_2 Apparatus.

Wash cup and inside of apparatus with 1% ammonia water colored with phenolphthalein to remove all acid.

Then rinse thoroughly with distilled water 3 times.

Expel all the water in the apparatus at outlet A (Fig. 29, Illus. I).

Seal the outlet and cup with mercury and check the apparatus in the following manner to see that it is airtight.

Close stopcock A and open B so that the mercury will fall through bulb B.

Lower the leveling bulb C until the mercury reaches the lower part of bulb A.

Reverse the stopcock B so that the mercury will rise through tube B.

Place leveling bulb C in upper ring (Position 1) until the mercury rises to stopcock A.

Expel any water or air that is present through outlet A.

Repeat the process until there is a metallic click produced by the mercury hitting stopcock A; this is evidence that a good vacuum has been produced. Evacuate the apparatus repeatedly until the metallic click is obtained 3 successive times.

aturation of Serum with Alveolar Air.

Place 2 or 3 cc. of serum in a 125 cc. separatory funnel.

. Fill the funnel with alveolar air by blowing into the funnel through a bottle of glass beads. (See Fig. 29, Illus. 2.)

  1) The operator makes as complete an expiration as possible after a normal inspiration.
  2) The funnel is closed with the stopper just before expiration is completed so that air will not be drawn back into the funnel.

. Rotate the funnel for 2 minutes spreading a film of serum over the bulb of the funnel.

. Repeat the expiration and rotating process.

etermination of CO_2.

. Place 0.5 cc. of water in the cup.

. Layer below it 1 cc. of serum which has been saturated with carbon dioxide, using a 1 cc. Mohr pipette not graduated to the tip or a volumetric pipette. (Do not draw the serum into the pipette more than once unless the serum is resaturated with CO_2 and do not use a "blow-out" pipette.)

c. Open stopcock B into bulb B, place leveling bulb C in the lower ring (Position 2), then open stopcock A allowing the serum and part of the water to slowly enter tube A.

d. Close stopcock A, taking care that no air enters the apparatus.

e. Wash the cup with 0.5 cc. of water and introduce most of it into tube A.

f. Place 2 drops of caprylic alcohol in the cup and introduce into tube A.

g. Place 0.5 cc. of 5% sulfuric acid in the cup and introduce into tube A.

h. Add 1 cc. of water to the cup and let sufficient amount into tube A to bring the mercury level to exactly 2.5 cc. (the highest point of the mercury meniscus).

i. Place mercury in the cup and seal the stopcock by allowing a few drops of mercury to run into the opening of stopcock A.

j. Remove excess fluid from cup and stopper.

k. Lower bulb C to position 3 or until the mercury in bulb A drops to the 50 cc. mark and then close stopcock B.

l. Remove the apparatus from the rack and mix the solutions by inverting the apparatus 20 times. (Some machines have a mechanical shaker attached to them.)

m. Replace the apparatus in the rack and allow 30 seconds to elapse for fluid to drain from the walls.

n. Open stopcock B and allow the fluid to flow into bulb B until the meniscus of the fluid is approximately at the 50 cc. mark. Do not allow any gas to leave bulb A.

o. Reverse stopcock B and bring the mercury back into bulb A through tube B.

p. Adjust the leveling bulb C so that the level of the mercury in it is 1/13 of the height of the fluid in tube A above the level of the mercury in tube A. (Fig. 29, Illus. 3.) This is an approximate correction for the specific gravity of the water column.

q. Read off the volume of carbon dioxide in tube A.

r. Note room temperature and the atmospheric pressure.

s. *Calculation:*
Correct the volume of carbon dioxide for standard atmospheric pressure by multiplying the volume by the pressure correction factor obtained from Table 82. Correct this value for standard temperature by reading it directly from Table 83, page 298. For milliequivalents divide volumes per cent by 2.22.

TABLE 82. CORRECTION FACTORS FOR BAROMETER READING (from Van Slyke and Stadie)

Barometer Reading	$\frac{B}{760}$	Barometer Reading	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.005
742	0.976	766	1.008
744	0.979	768	1.011
746	0.982	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

D. *Interpretation of Blood CO_2 Capacity.*

1. *Normal Values:*
   a. Adults: 53—70 volumes per cent or 24—32 milliequivalents.
   b. Infants: 40—55 volumes per cent or 18—25 milliequivalents.
2. *Alkalosis:*
   a. Above 70 volumes per cent.
   b. Due to excessive alkali therapy, oxygen therapy, hypercorticoadrenalism, typhus fever, pyloric obstruction, congenital intestinal alkalosis, and emphysema and other respiratory conditions.
3. *Acidosis:*
   a. Mild acidosis: 53—40 volumes per cent.
   b. Moderate acidosis: 40—30 volumes per cent.
   c. Severe acidosis: less than 30 volumes per cent.
   d. Found in diabetes, nephritis, severe diarrhea, hemorrhage, eclampsia, renal rickets, and many toxic conditions; also occurs after severe exercise, excessive intake of acidifying salts, and anesthesia.

Inorganic Constituents

I. Chlorides (Wilson and Ball's Method).

A. *Principle:* The chlorides are precipitated as silver chloride from serum with silver nitrate in the presence of nitric acid. The mixture is heated to digest the protein. The excess silver nitrate is determined by titration with ammonium thiocyanate using ferric alum [$FeNH_4(SO_4)_2 \cdot 12H_2O$] as an indicator.

$NaCl + AgNO_3 \rightarrow AgCl \downarrow + NaNO_3.$
$AgNO_3 + NH_4SCN \rightarrow AgSCN + NH_4NO_3.$
$3\ NH_4SCN + FeNH_4(SO_4)_2 \rightarrow Fe(SCN)_3 +$
$2\ (NH_4)_2SO_4.$

B. *General Considerations.*

1. Do not use a tourniquet when drawing the blood; if it is required to locate the vein, release before withdrawing the blood because venous stasis decreases serum chlorides.
2. The chlorides must be determined within 30 minutes after the blood is drawn.
3. Generally the serum chlorides increase as the CO_2 combining power decreases and vice versa.
4. Continued administration of large amounts of sodium bicarbonate will result in an increased CO_2 combining power and a compensatory fall in chlorides.
5. Acidifying chlorides (NH_4Cl, $MgCl_2$, and $CaCl_2$) used as diuretics will increase the serum chlorides due to the free HCl liberated by these salts.
6. *Sources of Error.*
   a. Inaccurate pipetting of the silver nitrate solution.
   b. Inaccurate titration with the thiocyanate solution.
7. If bromides are being given, see blood bromide determination on page 325.

C. *Method.*

1. Do test in duplicate.
2. Place 1 cc. of serum in large pyrex test tube.
3. Add exactly 1 cc. of standard silver nitrate solution (1 cc. ≎ 10 mg. NaCl) dropwise while shaking the tube.
4. Add 3 cc. of conc. nitric acid and heat over a microburner until all the protein is digested (solution above the AgCl becomes clear and light yellow in about 15 minutes).
5. Add 0.3 gm. of powdered ferric alum and wash down sides of tube with 6 cc. of water.
6. Cool to room temperature or lower; the colder the solution the sharper the endpoint in the titration.
7. Using a microburette, titrate with ammonium thiocyanate solution until the salmon pink color persists with shaking for 15 seconds.

   10 cc. NH_4SCN solution ≎ 1 cc. $AgNO_3$ solution ≎ 10 mg. of chlorides as NaCl.
8. *Calculation:*

   (10 — cc. of thiocyanate solution used) × 100 = mg. % of chlorides as NaCl.

   To express results in terms of milliequivalents of chloride per liter, calculate as above and divide the results by 5.85.

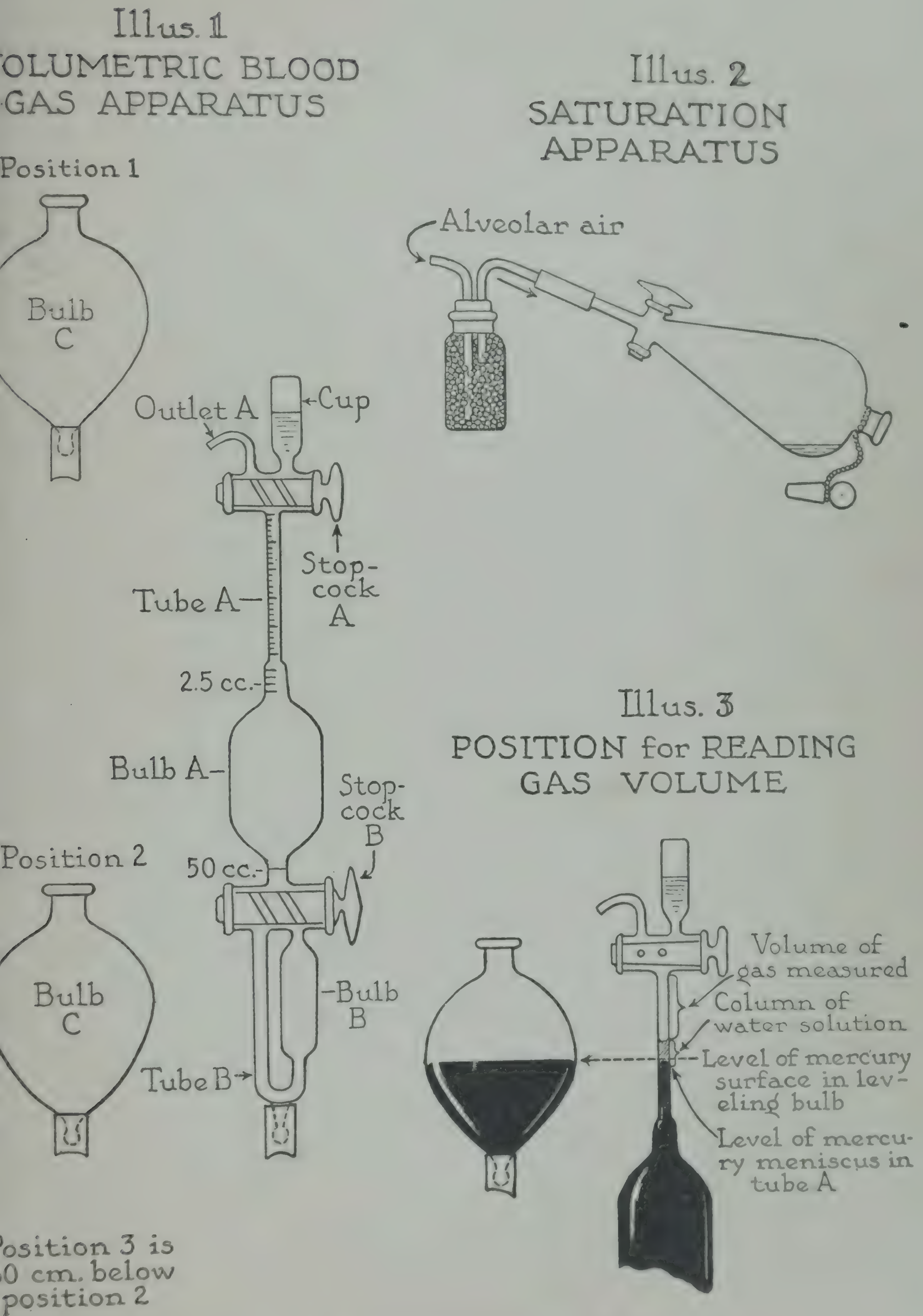

Illus. 1
OLUMETRIC BLOOD
GAS APPARATUS
Position 1
Bulb C
Outlet A
Cup
Stop-cock A
Tube A
2.5 cc.
Bulb A
Stop-cock B
Position 2
50 cc.
Bulb C
Bulb B
Tube B
osition 3 is
0 cm. below
position 2
Illus. 2
SATURATION APPARATUS
Alveolar air
Illus. 3
POSITION for READING GAS VOLUME
Volume of gas measured
Column of water solution
Level of mercury surface in leveling bulb
Level of mercury meniscus in tube A

Fig. 29. Apparatus for Gas Analysis.

TABLE 83. CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA (from Van Slyke and Cullen)

Observed Vol. gas $\times \frac{B}{760}$	cc. of CO_2 reduced to 0°C and 760 mm. pressure bound as bicarbonate by 100 cc. of plasma or serum			
	15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8
1	10.1	10.9	11.7	12.6
2	11.0	11.8	12.6	13.5
3	12.0	12.8	13.6	14.3
4	13.0	13.7	14.5	15.2
5	13.9	14.7	15.5	16.1
6	14.9	15.7	16.4	17.0
7	15.9	16.6	17.4	18.0
8	16.8	17.6	18.3	18.9
9	17.8	18.5	19.2	19.8
0.30	18.8	19.5	20.2	20.8
1	19.7	20.4	21.1	21.7
2	20.7	21.4	22.1	22.6
3	21.7	22.3	23.0	23.5
4	22.6	23.3	24.0	24.5
5	23.6	24.2	24.9	25.4
6	24.6	25.2	25.8	26.3
7	25.5	26.2	26.8	27.3
8	26.5	27.1	27.7	28.2
9	27.5	28.1	28.7	29.1
0.40	28.4	29.0	29.6	30.0
1	29.4	30.0	30.5	31.0
2	30.3	30.9	31.5	31.9
3	31.3	31.9	32.4	32.8
4	32.3	32.8	33.4	33.8
5	33.2	33.8	34.3	34.7
6	34.2	34.7	35.3	35.6
7	35.2	35.7	36.2	36.5
8	36.1	36.6	37.2	37.4
9	37.1	37.6	38.1	38.4
0.50	38.1	38.5	39.0	39.3
1	39.1	39.5	40.0	40.3
2	40.0	40.4	40.9	41.2
3	41.0	41.4	41.9	42.1
4	42.0	42.4	42.8	43.0
5	42.9	43.3	43.8	43.9
6	43.9	44.3	44.7	44.9
7	44.9	45.3	45.7	45.8
8	45.8	46.2	46.6	46.7
9	46.8	47.1	47.5	47.6
.60	47.7	48.1	48.5	48.6
0.60	47.7	48.1	48.5	48.6
1	48.7	49.0	49.4	49.5
2	49.7	50.0	50.4	50.4
3	50.7	51.0	51.3	51.4
4	51.6	51.9	52.2	52.3
5	52.6	52.8	53.2	53.2
6	53.6	53.8	54.1	54.1
7	54.5	54.8	55.1	55.1
8	55.5	55.7	56.0	56.0
9	56.5	56.7	57.0	56.9
0.70	57.4	57.6	57.9	57.9
1	58.4	58.6	58.9	58.8
2	59.4	59.5	59.8	59.7
3	60.3	60.5	60.7	60.6
4	61.3	61.4	61.7	61.6
5	62.3	62.4	62.6	62.5
6	63.2	63.3	63.6	63.4
7	64.2	64.3	64.5	64.3
8	65.2	65.3	65.5	65.3
9	66.1	66.2	66.4	66.2
0.80	67.1	67.2	67.3	67.1
1	68.1	68.1	68.3	68.0
2	69.0	69.1	69.2	69.0
3	70.0	70.0	70.2	69.9
4	71.0	71.0	71.1	70.8
5	71.9	72.0	72.1	71.8
6	72.9	72.9	73.0	72.7
7	73.9	73.9	74.0	73.6
8	74.8	74.8	74.9	74.5
9	75.8	75.8	75.8	75.4
0.90	76.8	76.7	76.8	76.4
1	77.8	77.7	77.7	77.3
2	78.7	78.8	78.7	78.2
3	79.7	79.6	79.6	79.2
4	80.7	80.5	80.6	80.1
5	81.6	81.5	81.5	81.0
6	82.6	82.5	82.4	82.0
7	83.6	83.4	83.4	82.9
8	84.5	84.4	84.3	83.8
9	85.5	85.3	85.2	84.8
1.00	86.5	86.2	86.2	85.7

D. *Solutions.*

1. *Standard Silver Nitrate Solution.*
   a. Weigh accurately 29.061 gm. of fused silver nitrate ($AgNO_3$), dissolve in water in a liter volumetric flask, and make to volume with water (1 cc. ≎ 10 mg. NaCl).
   b. Keep in a glass-stoppered brown bottle.
2. *Ammonium Thiocyanate Solution.*
   a. Dissolve 1.4 gm. of c.p. ammonium thiocyanate (NH_4SCN) in a liter of water.
   b. Titrate the thiocyanate solution with 1 cc. of the silver nitrate solution in the same manner as the serum chlorides are determined.
   c. Adjust the volume of the thiocyanate solution so that 10 cc. of it are equivalent to 1 cc. of the standard silver nitrate solution.
3. *Ferric Alum* [$FeNH_4(SO_4)_2 \cdot 12H_2O$] must be white after being finely powdered; if yellow it can not be used.

E. *Interpretation of Serum Chloride Findings.*

1. *Normal Values:* 580-630 mg. per cent as NaCl or 99-108 milliequivalents of chloride per liter.
2. *Increased in:*
   Hypoproteinemia
   Cardiac decompensation
   Hyperventilation (due to loss of CO_2)
   Nephroses
   Serum sickness
   Anemia
   Prostatic and other types of urinary obstruction.
3. *Decreased in:*
   Extensive burns
   Tetany (bicarbonate)
   Excessive vomiting
   Intestinal obstruction
   Diarrhea
   Eclampsia (with vomiting)

eat cramps
chloride of mercury poisoning
iabetic acidosis
mphysema
ther anesthesia
remia
obar pneumonia
dvanced nephritis
xcessive perspiration
evers—acute infections
ddison's disease
yphus fever
ypercorticoadrenalism
ongenital intestinal alkalosis
eneralized edema
naphylactic shock

II. Spinal Fluid Chlorides.

rocedure is the same as for serum chlorides xcept 1 cc. of spinal fluid is used in place of erum.

nterpretation.

ee Table 26 on page 154.

or correct interpretation of spinal fluid chlorides, blood should be drawn at the same time he spinal fluid is obtained because they vary n direct ratio to each other.

n increase in total proteins of the spinal fluid iminishes the spinal fluid chlorides.

III. Urine Chlorides (Volhard-Harvey Method).

rinciple: Chlorides are precipitated as silver hloride from the urine using silver nitrate nd nitric acid. The nitric acid prevents the recipitation of silver phosphate. The excess ilver nitrate is titrated with ammonium thiocyanate using ferric alum as an indicator.

General Consideration.

f the urine is alkaline, acidify with glacial cetic acid because silver nitrate will form a ark-colored precipitate in alkaline urine.

Protein must be removed by acidifying, heating, and filtering because it combines with silver nitrate giving inaccurate results for chlorides.

Diacetic acid in the urine will give a red color with ferric alum before any ammonium thiocyanate solution is added. The diacetic acid can be removed by acidifying the urine and boiling it for a few minutes.

The patient should not be given salicylates (aspirin) before collecting the urine because the urine will give a red color with ferric alum. This may be dispelled by adding a few drops of a saturated solution of potassium permanganate.

C. *Method.*

1. Place 5 cc. of urine in a 125 cc. Erlenmeyer flask and add 15 cc. of water.
2. Add 5 cc. of standard silver nitrate solution (1 cc. $\Bumpeq$ 10 mg. NaCl) and mix.
3. Add 0.7 cc. of conc. nitric acid, mix, and allow to stand for 10 minutes in the dark.
4. Add 0.3 gm. of powdered ferric alum and 2 drops of caprylic alcohol; titrate with ammonium thiocyanate solution until a salmon pink color is formed which persists with shaking for 15 seconds.

   1 cc. NH_4SCN solution $\Bumpeq$ 1 cc. $AgNO_3$ solution $\Bumpeq$ 10 mg. NaCl.
5. If the end-point is obtained immediately on adding the thiocyanate, add another 5 cc. of silver nitrate and titrate, changing the calculation accordingly.
6. *Calculation:*

$$(5 - \text{titration}) \times 10\text{ mg.} \times \frac{100}{5} = \text{mg.\% of chlorides as NaCl.}$$

D. *Solutions.*

1. *Standard Silver Nitrate Solution*—see blood chlorides.
2. *Ammonium Thiocyanate Solution.*
   a. Dissolve 13 gm. of ammonium thiocyanate (NH_4SCN) in a liter of water.
   b. Place 10 cc. of the standard silver nitrate solution in an Erlenmeyer flask and add 40 cc. of water and 1.5 cc. of conc. nitric acid.
   c. Add 1 gm. of powdered ferric alum and titrate with the ammonium thiocyanate solution until a permanent red-brown tinge is produced.
   d. Adjust the volume of thiocyanate solution so that 10 cc. are equivalent to 10 cc. of the standard silver nitrate solution.

E. *Interpretation of Urine Chloride Findings.*

1. *Normal Values:* 0.7—1 gm. per cent as NaCl or 10—15 gm. in 24 hours.
   a. The amount varies with the chlorides ingested.
   b. Fasting and strenuous physical exercise decreases the chlorides.
   c. Excessive water intake increases the chlorides.
2. *Decreased in:*
   Severe diarrhea
   Congenital intestinal alkalosis
   Nephritis with edema
   Diabetes
   Acute infections, especially those with formation of an exudate.
   Hypertension
   Pneumonia

Extensive burns
Anemia
Carcinoma of the stomach

IV. Wilder's Test for Addison's Disease (Cutler, Power, and Wilder).

A. ***Principle:*** After being on a salt-free diet and a measured amount of fluid intake for 3 days, a patient with Addison's disease will excrete an increased amount of chlorides.

B. ***Outline of Test.***

1. The patient is kept on a salt-free diet for 3 days with the following fluid intake.
   a. The first day there is no restriction of fluid.
   b. The second day the fluid intake should amount to 40 cc. per kilogram of body weight.
   c. The third day 20 cc. of fluid per kilogram of body weight is given before 11:00 A. M.
2. On the third morning (8 to 12 A. M.) a 4 hour specimen of urine is collected.
3. Determine the mg. per cent of chlorides as NaCl in the urine (see method for urine chlorides).

C. ***Interpretation.***

1. *Normal Value* for 4 hour specimen: 30—250 mg. per cent NaCl.
2. *Increased in:*
   Addison's disease
   Hypopituitarism

V. Kepler's Test for Addison's Disease (Robinson, Power, and Kepler).

A. ***Part I.***

1. On the day before the test the patient eats 3 ordinary meals but omits extra salt.
2. No food or fluid should be taken after 6 o'clock in the evening.
3. At 10:30 P. M. the patient empties his bladder and the urine is discarded.
4. Pool all urine voided from 10:30 P.M. to and including 7:30 A.M.; measure the volume and save. This is the night urine.
5. A specimen is collected at 8:30 A.M. and discarded.
6. Immediately after this give the patient 20 cc. of water per kilogram of body weight (9 cc. per lb.) which he drinks within the next 45 minutes.
7. At 9:30, 10:30, 11:30, and 12:30, collect urine specimens in separate containers, label as to time, and save.
8. The patient should rest in bed except to collect specimens.
9. The volume of the largest specimen is measured.
10. If the patient is unable to void at any period, then calculate the amount of urine excreted per hour.
11. *Conclusion:*
    a. If the volume of any single hourly specimen voided during the morning is greater than the total volume of urine voided during the night, the test is negative and the patient does not have Addison's disease; therefore, the test is discontinued.
    b. If the volume of the largest hourly specimen voided in the morning is less than the volume of the night specimen, the test is positive and the patient may or may not have Addison's disease; therefore, the test must be continued and Part II carried out.

B. ***Part II.***

1. While the patient is still fasting, obtain 10 cc. of venous blood and place in a bottle containing lithium oxalate.
2. Determine the following:
   a. Plasma urea N (mg.%).
   b. Plasma chlorides (mg.%).
   c. Urine urea N (mg.%) in the night specimen.
   d. Urine chlorides (mg.%) in the night specimen.
3. *Equation:*

$$A = \frac{\text{Urea N in urine}}{\text{Urea N in plasma}} \times \frac{\text{Chlorides in plasma}}{\text{Chlorides in urine}} \times \frac{{}^{*}\text{Vol. day urine (cc.)}}{\text{Vol. night urine (cc.)}}$$

*Volume of largest 1 hour specimen.

4. *Conclusion:*
   a. If A is greater than 30, the patient probably does not have Addison's disease.
   b. If A is less than 25, the patient probably has Addison's disease provided that nephritis has been excluded.
   c. If A is between 25 and 30, the test is doubtful and the Wilder test should be done.

VI. Sodium (Butler and Tuthill's Method).

A. ***Principle:*** The sodium is quantitatively precipitated with uranyl zinc acetate and gravimetrically determined as uranyl zinc sodium acetate, $(UO_2)_3ZnNa(CH_3COO)_9 \cdot 6H_2O$ (mol. wt. = 1538).

B. ***General Considerations.***

1. All glassware is washed only with hot water

d rinsed with distilled water. It is never eaned with cleaning solution.

na filter crucibles, fritted glass, porosity o. 4, holding about 35 cc. are used.

After a determination is completed, scrape the uranyl zinc sodium acetate from the filter into a bottle and keep to make the saturated alcoholic solution.

Wash filters with hot water, distilled water, then dry with alcohol and ether, using suction.

Keep filters in a desiccator over calcium chloride when not in use.

One hour before weighing, heat filters in an oven (not over 110°C.) for 30 minutes and then replace in the desiccator to come to a constant weight.

ources of Error.

Not placing the glass bead used in the test in the filter for the original weighing.

The wash solution not being saturated with the triple salt and filtered just before using.

The precipitate in the filter not being thoroughly dry before weighing.

Method.

Test is done in triplicate.

Pipette 1 cc. of serum into a thick walled Pyrex test tube (200 × 25 mm.).

Add one glass bead, 1 cc. of 4 N sulfuric acid, and 0.5 cc. of conc. nitric acid.

Digest as for Total Protein using a very low flame and watching carefully to avoid cooking to dryness.

When charring appears, remove from the flame and add carefully 1 drop of 30% hydrogen peroxide (superoxyl) directly into the solution.

Digest 3 or 4 minutes longer and add another drop of hydrogen peroxide; repeat this process until the solution is clear (usually takes 3 to 5 drops of hydrogen peroxide).

Continue heating for a few minutes after the solution is clear.

Cool, add 4 to 5 drops of water, and then pour quantitatively into approximately 15 cc. of freshly filtered uranium zinc acetate reagent (should be shaken frequently preceding use to insure saturation) in a weighed Jena glass filter which is fitted with a solid rubber stopper from below. The stopper prevents the liquid from going through the filter.

Rinse the contents of the test tube into the filter with three 0.5 cc. portions of water and finally three 2 cc. portions of reagent. If there is any sodium left in the test tube, it will show as a precipitate.

10. Stir the solution in the filter until a precipitate appears and for several minutes thereafter.
11. Withdraw the stirring rod, rinsing it with 3 cc. of reagent as it is withdrawn.
12. Cover the filter with a watch glass and let it stand at room temperature for 1 hour.
13. Remove the rubber stopper, place the filter in a suction flask, and apply suction.
14. After the reagent has been filtered off, wash the precipitate with five 2 cc. portions of freshly filtered 95% alcohol saturated with the triple salt (uranyl zinc sodium acetate). Care should be taken to wash down the sides of the filter. Delay in the washing makes this more difficult.
15. Finally wash with two 5 cc. portions of ether.
16. Continue suction until the precipitate is thoroughly dry.
17. Remove filter from the suction flask and wipe off the outside and bottom of the filter with a cloth soaked in ether.
18. Place the filter in the desiccator and weigh after 1 hour.
19. *Calculation:*

    The precipitate contains 1.495% sodium

$$\left(\frac{23}{1538} \times 100 = 1.495\right).$$

$$\text{Wt. of ppt.} \times 0.01495 \times \frac{100}{\text{cc. of serum}} = \text{mg.\%.}$$

D. *Solutions.*

1. Saturated solution of precipitated uranyl zinc sodium acetate (obtained from filters) in 95% alcohol.
   a. About 0.5 gm. per 100 cc. of alcohol.
   b. Filter just before using.
   c. The salt is initially prepared as follows:
      1) Place 20 cc. of the uranium zinc acetate reagent in a beaker and add 3 cc. of 0.85% NaCl solution.
      2) Stir until a precipitate is formed and filter through Whatman No. 50 filter paper.
      3) Wash the precipitate (uranyl zinc sodium acetate) on the filter paper with cold water 4 or 5 times, then allow to dry.
2. *Uranium Zinc Acetate Reagent.*
   a. *Solution A.*
      1) Weigh a liter Erlenmeyer flask and add 80 gm. of sodium free uranyl acetate $[UO_2(C_2H_3O_2)_2 \cdot 2H_2O]$.
      2) Add 48 gm. or 46 cc. of 30% acetic acid (per cent by volume) and make up to 520 gm. with water.

b. *Solution B.*
    1) Weigh a liter Erlenmeyer flask and add 220 gm. of zinc acetate [$Zn(C_2H_3O_2)_2 \cdot 2H_2O$].
    2) Add 24 gm. or 23 cc. of 30% acetic acid and make up to 520 gm. with water.

c. Cover and warm both Solutions (A and B) on a steam bath with occasional stirring or shaking until the chemicals are completely dissolved.

d. Mix while hot and let stand 24 hours before using.

e. If no yellow precipitate appears, add 0.2 gm. of precipitated uranyl zinc sodium acetate in order to saturate with this triple salt.

f. Shake the mixture several times just before using and filter to assure saturation at the temperature of the analysis.

g. Solution keeps indefinitely.

3. *Sulfuric Acid*—4 N.
    a. Add 112 cc. of conc. H_2SO_4 to 600 cc. of water in a liter volumetric flask.
    b. Cool and dilute to volume with water.

E. *Interpretation of Serum Sodium Findings.*

1. *Normal Values:* 325—350 mg. per cent or 141—152 milliequivalents.
2. *Decreased in:*
    Addison's disease
    Myxedema
    Dehydration
    Severe burns
    Sprue
3. *Increased in:*
    Pyloric obstruction
    Terminal nephritis
    Hypercorticoadrenalism

VII. Potassium (Weichselbaum, Somogyi, and Rush's Method).

A. *Principle:* Potassium in serum is precipitated as potassium silver cobalt nitrite by using a slightly modified tungstic acid filtrate from which both proteins and chlorides have been removed. The precipitate is dissolved in dilute nitric acid and the silver is titrated with potassium thiocyanate using ferric alum as an indicator.

B. *General Considerations.*

1. There must not be any hemolysis of the blood.
2. After withdrawing the blood from the vein, it should stand not longer than 20 minutes before the serum is separated from the clot by centrifugation.
3. The serum can be kept in the refrigerator until time to do the test.
4. Clean all glassware with nitric acid before using, rinsing well with water.
5. *Sources of Error.*
    a. Correct ratio of silver ion to nitrite is important to prevent formation of silver nitrite.
    b. Sodium cobalt nitrite reagent decomposes at temperatures higher than 20°C.
    c. Care should be taken not to break up the precipitate while washing.
    d. The end-point is so sharp that one should be able to duplicate the titration with a maximum error of 0.01 cc.

C. *Method.*

1. Pipette 2 cc. of serum into a pyrex test tube containing 12.4 cc. of water and mix well. (If a potassium below 15 mg. is expected, use 3 cc. of serum making a 1-7.5 dilution, or 4 cc. making a 1-5 dilution. If a high potassium is expected, use 1.5 cc. or 1 cc. of serum.)
2. Add 2 cc. of 3% sodium tungstate solution and mix.
3. Add 2 cc. of 5% copper sulfate solution, stopper, and shake well.
4. Add 1.6 cc. of 0.2 M silver nitrate solution, stopper, and shake well.
5. Filter through 9 cm. filter paper (Whatman No. 40), returning first part of filtrate if not clear.
6. Set up the following procedure in duplicate.
    a. Pipette 5 cc. of the filtrate into a 15 cc. conical tipped centrifuge tube.
    b. Add 0.5 cc. of 0.2 M silver nitrate solution and mix contents with a fine glass rod. (If a 1-5 dilution of serum is used, add only 0.45 cc. of silver nitrate solution.)
    c. Place the tubes in an 18-20°C. water bath.
    d. When the tubes assume the temperature of the water bath, add dropwise with stirring 0.5 cc. of a freshly prepared 12.5% solution of sodium cobalt nitrite which has been cooled to 18-20°C.
    e. Let stand in the water bath for 10 to 20 minutes and then centrifuge at 3000 revolutions per minute.
    f. Using suction, remove the supernatant fluid by means of a finely drawn glass tube with the tip curved upward.
    g. When the tubes are not in the centrifuge or in manipulation, keep in the water bath.
    h. Wash the precipitate 3 times with 5 cc. portions of water (18-20°C.).
        1) Hold the tube in a slanting position (25° angle) and rotate while about 1 cc. of the first portion of water is

allowed to run dropwise and slowly down the side of the tube, the rest is allowed to run in rapidly.

2) Centrifuge after each washing and remove supernatant fluid as described above.

Cover the tubes with tin foil after the ast washing and put in the refrigerator until ready to titrate.

Add 0.5 cc. of a 1—3 nitric acid solution and place in a boiling water bath for 5 minutes. If the precipitate does not go into solution, carefully agitate.

Let tubes cool and add 1 drop of 20% ferric alum solution.

Titrate to a faint but permanent pink with 0.0025 M potassium thiocyanate solution from a microburette.

0.1 mg. K ≎ 0.276 mg. Ag ≎ 1.06 cc. of 0.0025 M KSCN solution.

Calculation:

$$\frac{\text{cc. used in titration} \times 0.1 \times 100}{1.06 \times 0.5} =$$

$$\text{cc. used in titration} \times 18.87 = \text{mg. \%.}$$

utions.

ium Cobalt Nitrite Solution—12.5%.

Place 150 gm. of sodium nitrite ($NaNO_2$) in a liter Florence flask and dissolve in 150 cc. of hot water.

Cool to about 40°C. and add 50 gm. of cobalt nitrate crystals [$Co(NO_3)_2 \cdot 6H_2O$] with vigorous stirring until dissolved.

Add 50 cc. of 50% acetic acid in approximately 2 cc. portions with whirling after each addition. After the addition of 25 cc. and after the entire 50 cc. have been added, the flask is stoppered and vigorously shaken.

The yellow-brown precipitate is filtered off and discarded.

After aeration for 45 minutes with a rapid current of air, the filtrate is allowed to stand in the refrigerator overnight.

The supernatant fluid is then passed through a dry filter paper and the brown precipitate is again discarded.

To the filtrate add in small portions about 200 cc. of 95% alcohol with vigorous agitation.

Allow to stand 30 minutes and then filter through a Buchner funnel with suction. The precipitate is washed with 4 successive 25 cc. portions of 95% alcohol, followed by three washings with pure ether.

Place the precipitate in a desiccator to dry.

   k. Grind in an agate mortar and store in a glass-stoppered brown bottle.
   l. The dry salt is stable.

2. *Sodium Tungstate Solution*—3%.
3. *Copper Sulfate ($CuSO_4 \cdot 5H_2O$) Solution*—5%.
4. *Silver Nitrate Solution* (0.2 M)—place 3.4 gm. of silver nitrate ($AgNO_3$) in a 100 cc. volumetric flask and make up to volume with water.
5. *Nitric Acid* (1—3)—add 1 volume of nitric acid to 2 volumes of water.
6. *Ferric Alum Solution*—20%.
   a. Place 20 gm. of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in a 100 cc. volumetric flask containing about 50 cc. of water.
   b. Add 3 cc. of nitric acid and dilute to volume with water.
7. *Potassium Thiocyanate Solution.*
   a. *Stock Solution*—0.1 M.
      1) Place 9.717 gm. of potassium thiocyanate (KSCN) in a liter volumetric flask.
      2) Make up to volume with water.
   b. *Dilute Solution*—0.0025 M.
      1) Pipette 5 cc. of the stock solution into a 200 cc. volumetric flask and dilute to volume with water.
      2) Keeps 2 days.

E. *Interpretation of Serum Potassium Findings.*

1. *Normal Values:* 18—21 mg. per cent or 4.6—5.6 milliequivalents.
2. *Increased in:*
   Addison's disease
   Pneumonia
   Acute infections
   Uremia
   Acute bronchial asthma
3. *Decreased in:*
   Hereditary periodic paralysis
   Hypercorticoadrenalism
   Overdosage of desoxycorticosterone
   Overdosage of testosterone
   Congenital intestinal alkalosis
   Severe acute diarrhea
   Chronic nephritis
   Hyperinsulinism and diabetes
   Malignant growths
   Sprue and chronic diarrhea

VIII. Calcium (Clark-Collip Modification of the Kramer-Tisdall Method).

A. ***Principle:*** Serum calcium is precipitated directly as calcium oxalate. The oxalic acid is then released by sulfuric acid and titrated with a standard solution of potassium permanganate.

$CaC_2O_4 + H_2SO_4 \rightarrow CaSO_4 + H_2C_2O_4$.

$5\ H_2C_2O_4 + 2\ KMnO_4 + 3\ H_2SO_4 \rightarrow 2\ MnSO_4 + K_2SO_4 + 10\ CO_2 \uparrow + 8\ H_2O$.

B. *General Considerations.*

1. The blood should be centrifuged and the serum separated from the cells within 30 minutes after withdrawal from the patient. On standing the cells become permeable to the calcium lowering the result.
2. Centrifuge tubes should be cleaned by heating a few minutes in a concentrated cleaning solution.
3. *Sources of error.*
   a. All the excess oxalate must be removed by washing with ammonium hydroxide or too high results will be obtained.
   b. There must not be any precipitate in the 4% ammonium oxalate.
   c. Careless draining of the tubes.
   d. Allowing the temperature of the solution to drop below 70°C. during the titration.

C. *Method.*

1. Test must be done in duplicate.
2. Place 2 cc. of serum in a pyrex centrifuge tube which has an outside diameter of 6-7 mm. at the 0.1 cc. mark.
3. Add 2 cc. of water and mix contents of the tube by rotating between the hands.
4. Slowly add 1 cc. of 4% ammonium oxalate solution, shaking while adding, and mix as above.
5. Stopper and allow to stand overnight or at least 3 hours in the refrigerator.
6. Mix again and centrifuge for 15 minutes.
7. Pour off the supernatant fluid carefully; keeping the tube inverted, place it in a rack with the mouth resting on a piece of filter paper. Allow to drain 5 minutes and then wipe the mouth of the tube dry with Whatman No. 40 filter paper.
8. Add 3 cc. of 2% ammonium hydroxide by blowing in approximately 2 cc. from a fine tipped volumetric pipette and washing the sides of the tube with the remainder.
9. Mix thoroughly by rotating in the hands and then centrifuge.
10. Repeat procedure outlined in 7, 8, and 9 and again pour off the supernatant fluid and drain as described in 7.
11. Add 2 cc. of approximately normal sulfuric acid by blowing it from a pipette directly upon the precipitate so as to break up the mat and facilitate solution.
12. Place tube in a boiling water bath for 1 minute.
13. Keep tubes in water bath at 70-75°C. while titrating with 0.01 N potassium permanganate solution.
14. Titrate from a microburette to a definite pink which persists for at least 1 minute.
15. Take temperature of water in water bath at end of titration to make sure it is not below 70°C.
16. *Calculation:*

1 cc. of 0.01 N $KMnO_4$ ≎ 0.2 mg. of calcium.

$$A \times 0.2 \times \frac{100}{2} = 10A = \text{mg. \%}.$$

A = number cc. of potassium permanganate solution used after correcting if solution is not 0.010 N. The correction is made by multiplying the cc. used by the normality factor.

D. *Solutions.*

1. *Potassium Permanganate Solution*—0.1 N.
   a. Dissolve 3.2 gm. of pure $KMnO_4$ in a liter of redistilled water in a thoroughly clean 2 liter Florence flask.
   b. Insert funnel covered with a watch glass as a condenser and digest for several hours at or near the boiling point.
   c. Cool, let stand overnight, and filter with gentle suction through a three inch Buchner filter lined with ignited asbestos or glass wool.
   d. Transfer the filtrate to a glass-stoppered brown bottle, keep in a dark place, and do not use until it has stood one month to become stabilized.
   e. It is not necessary to standardize this solution to make the 0.01 N solution.
2. *Potassium Permanganate Solution*—0.01 N.
   a. Make a 1-10 dilution of the 0.1 N solution and allow to stand one month to become stable before standardizing.
   b. *Standardization.*
      1) Make a 0.01 N sodium oxalate solution.
         a) Dry some pure anhydrous sodium oxalate ($Na_2C_2O_4$) in an oven at 100-105°C. for 12 hours.
         b) Dissolve exactly 0.67 gm. of the oxalate in redistilled water in a liter volumetric flask, add 5 cc. of conc. sulfuric acid, and dilute to volume.
      2) Transfer exactly 25 cc. of this solution to a 100 cc. Erlenmeyer flask.
      3) Add 1 cc. of conc. sulfuric acid, heat to 80°C., and titrate with the $KMnO_4$ solution until the addition of a single drop gives a pink color which persists for 1 minute. The temperature of the solution should not go below 70°C.

4) Calculate the normality of the $KMnO_4$ solution by dividing the cc. of sodium oxalate solution by the cc. of permanganate solution used in the titration and multiplying the quotient by 0.01. The factor is obtained by multiplying the normality by 100.

5) Check solution every week.

mmonium Oxalate Solution (4%) — filter quently to remove precipitate.

proximately Normal Sulfuric Acid.

Add 28 cc. conc. sulfuric acid to about 900 cc. of water in a liter volumetric flask.

Cool and dilute to volume with water.

erpretation of Serum Calcium Findings ee Table 85).

rmal Values:

ults: 9.5—11 mg. per cent or 4.7—5.5 liequivalents.

ildren: 10—11.5 mg. per cent or 5.0—5.8 liequivalents.

ants: 10.5—12.0 mg. per cent or 5.2—6.0 liequivalents.

Serum proteins influence the calcium level; it is increased in hyperproteinemia and decreased in hypoproteinemia.

In general there is a reciprocal relation between calcium and phosphorus; a change in one is followed by an opposite change in the other.

reased in:

yperparathyroidism
lycythemia vera
ypervitaminosis (excess vitamin D feeding)
ltiple myeloma
rcinoma (metastatic to bone)
urofibromatosis

creased in:

poparathyroidism
nal rickets
tany (infantile, parathyroid, rachitic, and tric)
teomalacia
tamin D deficiency
opathic steatorrhea
rue
liac disease
vere nephritis
ephrosis
eumonia
egnancy
emophilia calcipriva
ute pancreatic necrosis (between 3rd and th days)

. Urine Calcium (Wang's Method).

inciple: Urine is treated with trichloroacetic acid and charcoal to remove protein and chromogenic substances present. After clearing by filtration, the calcium is precipitated with ammonium oxalate and adjusted to a pH of 5. The calcium oxalate is washed and heated to 100°C. for one hour to remove the organic solvents. Sulfuric acid is added to the calcium oxalate and the oxalic acid released is titrated with a standard solution of potassium permanganate.

B. *General Considerations.*

1. Patient should be on a low calcium and neutral ash diet (no dairy products) for 3 days previous to collecting the specimen.
2. Normally a person should be in a positive calcium balance, that is, the calcium excreted in the urine and feces together should be less than the intake.
3. A 24 hour specimen of urine is necessary.
4. Check the charcoal for calcium by running a control using distilled water instead of urine. If there is any calcium present in the control, it must be subtracted from the urine calcium.

C. *Method.*

1. Mix the 24 hour specimen of urine thoroughly and measure the volume.
2. Place 100 cc. of the urine in a 250 cc. Erlenmeyer flask and add 25 cc. of 20% trichloroacetic acid.
3. Add 2 gm. of acid washed charcoal, stopper, shake, and allow to stand for 20 minutes.
4. Filter through Whatman No. 40 filter paper.
5. Do the following procedure in triplicate plus a control on the charcoal.
6. Place 5 cc. of the filtrate in each of three 15 cc. conical centrifuge tubes. (Use 10 cc. if the calcium value is expected to be low.)
7. Add 1 cc. of 20% sodium acetate solution and 6-8 drops of bromcresol green or methyl red.
8. Add 1 cc. of 0.1 M ammonium oxalate solution directly into the solution and mix by stirring with a glass rod.
9. Adjust to a pH of 5 (green if bromcresol green is used, pink if methyl red is used) with 10% ammonium hydroxide and 10% acetic acid.
10. Remove glass rod and rinse into the tube with water.
11. Allow the tubes to stand overnight in the refrigerator and then centrifuge for 15 minutes.

12. Decant carefully, leaving the tube in an inverted position to drain on a pad of filter paper for 5 minutes.
13. Wipe the mouth of the tube with a strip of Whatman No. 40 filter paper.
14. Wash and break up the clot of calcium oxalate by blowing in 3 cc. of 2% ammonium hydroxide in an alcohol, ether, and water mixture.
15. Centrifuge for 15 minutes, decant, and drain as above.
16. Repeat the washing and draining process.
17. Place tubes in an oven at 85-100°C. for 1 hour to remove all organic solvents.
18. Add 2 cc. of approximately 2 N sulfuric acid and place in a boiling water bath for 2 minutes.
19. Titrate with 0.01 N potassium permanganate solution at 70°C. to a faint pink which persists for 1 minute.
20. *Calculation:*

1 cc. of 0.01 N $KMnO_4$ ≎ 0.2 mg. of calcium.

$A \times 0.2 \times \frac{\text{cc. of urine in 24 hr.}}{4} =$ mg. of calcium excreted in 24 hours.

A = number of cc. of potassium permanganate solution used after correcting if solution is not 0.010 N. If the charcoal control tube contains any calcium, it must be subtracted from the amount obtained in the above calculation.

D. *Solutions.*

1. *Trichloroacetic Acid*—20%.
2. *Sodium Acetate Solution*—20%.
3. *Ammonium Oxalate Solution*—0.1 M.
    a. Dissolve 1.42 gm. of ammonium oxalate $[(NH_4)_2C_2O_4 \cdot H_2O]$ in water in a 100 cc. volumetric flask.
    b. Dilute to volume with water.
    c. Add a crystal of thymol and keep in the refrigerator.
4. *Ammonium Hydroxide*—10%.
5. *Acetic Acid*—10%.
6. *Washing Solution*—add 20 cc. of ammonium hydroxide to 980 cc. of a mixture of 1 volume of ether, 1 volume of alcohol, and 1 volume of water.
7. *Sulfuric Acid* (Approximately 2 N).
    a. Add 54 cc. of conc. sulfuric acid to 900 cc. of water in a liter volumetric flask.
    b. Cool and dilute to volume with water.
8. *Potassium Permanganate Solution*—0.01 N (see blood calcium).

E. *Interpretation of Urine Calcium Findings* (See Table 85).

1. *Normal Values:* 50—300 mg. per 24 hour specimen. (Range of 0.2 to 4.6 mg. per kg.)
2. *Increased in:*
    Hyperparathyroidism
    Hyperthyroidism
    Sprue
    Celiac disease
    Carcinoma (metastatic to bone)
    Hypervitaminosis (vit. D. therapy)
    Multiple myeloma
    Osteitis deformans (Paget's disease)
3. *Decreased in:*
    Hypoparathyroidism
    Hypothyroidism
    Idiopathic steatorrhea
    Osteomalacia
    Renal rickets
    Rickets
    Nephritis

X. Calcium in Feces (Wang's Method).

A. ***Principle:*** Feces is dried, finely ground, a digested with nitric-perchloric acid mixtu Calcium is determined on an aliquot porti using the method for urine calcium.

B. *General Considerations.*

1. Patient should be on a low calcium and neutr ash diet (no dairy products) for 3 days pr vious to collecting the specimen.
2. Feces formed during a definite time interv can be marked by giving a 5 gram capsu of carmine with the first meal of a metab lism period and again with the first me after the period. Save all specimens begi ning with the first containing carmine un the appearance of the second carmine.

C. *Method.*

1. Place the entire 24 hour fecal specimen in weighed evaporating dish, dry over a stea bath for 4 to 6 hours and then in a hot a oven (not over 100°C.) until complete dry (at least 24 hours).
2. Cool and weigh the evaporating dish wit the entire specimen.
3. Place about 1 gm. of the dried feces i a mortar and grind to a fine powder.
4. Weigh accurately (chemical balance) abou 0.5 gm. of the powder and place in a 800 cc. pyrex Kjeldahl flask.
5. Add 20 cc. of nitric-perchloric acid mixtur
6. Heat flask over a microburner with con stant shaking in order to avoid an *Explo sion.*
7. When white fumes appear and the liqui starts to darken, add 10-20 cc. more of th acid mixture.
8. Continue digestion until mixture become colorless or only slightly colored.

9. Cool flask and transfer quantitatively into a 500 cc. volumetric flask and dilute to volume with water.

0. Pipette 5 cc. into each of 2 conical tipped 15 cc. centrifuge tubes and proceed as for urine calcium.

1. *Calculation:*

$$A \times 0.2 \times \frac{500}{5} = \text{mg. of calcium in sample used.}$$

A = number of cc. of potassium permanganate used after correcting if normality is not 0.010 N.

$$\frac{\text{Wt. of 24 specimen}}{\text{Wt. of sample used}} \times \text{mg. calcium in sample} =$$

calcium excreted in 24 hours.

Solutions:

Nitric-Perchloric Acid Mixture.

- a. Place 20 cc. of fuming nitric acid (sp.gr. 1.49) into a 200 cc. pyrex Erlenmeyer flask.
- b. Add 10 cc. of 60% perchloric acid (sp. gr. 1.615) and 20 cc. of water and mix.

Other Solutions—same as under urine calcium.

Interpretation of Fecal Calcium Findings (See Table 85).

Normal Values: 0.4—0.8 gm. in 24 hours depending on the calcium intake.

An average of 70 (60—90) per cent of the total calcium excreted from the body is in the feces.

Increased in:

Hyperthyroidism
Nephritis
Idiopathic steatorrhea

Decreased in:

Osteomalacia

XI. Inorganic Phosphorus (Bodansky's Colorimeter Method).

Principle: Serum protein is precipitated by trichloroacetic acid. Phosphomolybdic acid is formed by the interaction of phosphorus and acid-molybdate. Stannous chloride is used as a reducing agent to produce the blue colored mixture (colloidal reduced oxides of molybdenum), the intensity of which is proportional to the quantity of phosphorus present.

General Considerations.

Avoid hemolysis of the blood, because it increases the inorganic phosphorus.

The serum must be separated from the cells as soon as possible after withdrawing the blood from the patient and the inorganic phosphorus determined without delay.

3. The ingestion of glucose lowers the inorganic phosphorus so the blood must be taken before the patient eats breakfast.
4. *Sources of error.*
   - a. Incorrect addition of reagents.
   - b. Molybdate solution having a yellow color due to incorrect preparation.
   - c. Presence of phosphorus in the water.
   - d. Plungers and cups of colorimeter not absolutely clean.

C. *Method.*

1. Pipette 9 cc. of 10% trichloroacetic acid into a 15 cc. test tube and add 1 cc. of serum.
2. Stopper, mix well, and let stand 20 minutes.
3. Filter through 9 cm. Whatman No. 40 filter paper until perfectly clear.
4. Place 6 cc. of the filtrate in a large test tube.
5. Place 6 cc. of the dilute standard phosphate solution (6 cc. = 0.02 mg. P) in each of 2 large test tubes.
6. Add the 2 following reagents consecutively to each tube as follows:
   - a. Two cc. of acid-molybdate mixture (made fresh and checked) and mix contents by tapping tube.
   - b. Blow in 2 cc. of dilute stannous chloride reagent (made fresh and checked), mixing during the addition.
7. The color develops rapidly and comparison with the standard in a colorimeter may be made immediately or any time within 2 hours.
8. Check standards against each other before making comparison with unknowns.
9. *Calculation:* The value in mg. per 100 cc. for the mm. reading on the colorimeter is found in Table 84 under the division corresponding to the volume (aliquots) used because inorganic phosphorus does not obey Beer's Law.

D. *Solutions Made Daily and Checked.*

1. *Preparation.*
   - a. *Acid-molybdate mixture.*
     1) Add quickly, while mixing, 1 volume of 7.5% sodium molybdate solution to 1 volume of cold 10 N sulfuric acid.
     2) The mixture should be free from even the slightest tinge of yellow.
   - b. *Dilute stannous chloride solution.*
     1) Dilute 0.5 cc. of a 60% stannous chloride solution to 200 cc. with water.
     2) Keep in the refrigerator between analyses if more than one series of determinations is to be done during the day.

TABLE 84. INORGANIC PHOSPHORUS CONTENT IN MILLIGRAMS PER 100 CC. CORRECTED FOR DEVIATION FROM BEER'S LAW

For 6 cc. aliquots (or for 3 cc. when half volumes are used).							For 4 cc. aliquots (or for 2 cc. when half volumes are used).						
Mm.	0.0	0.2	0.4	0.6	0.8	D	Mm.	0.0	0.2	0.4	0.6	0.8	D
Mm.	mg.	mg.	mg.	mg.	mg.		Mm.	mg.	mg.	mg.	mg.	mg.	
5	17.7	16.9	16.2	15.5	14.9	0.3+	5	25.8	24.7	23.7	22.7	21.8	0.5
6	14.4	13.8+	13.4	12.9	12.5	0.2+	6	20.9	20.1	19.4	18.8	18.2	0.3
7	12.0+	11.7	11.3	11.0	10.7	0.2−	7	17.6	17.1	16.6	16.1	15.6	0.2
8	10.4	10.1	9.8	9.6−	9.3	0.1+	8	15.1	14.7	14.3	13.9	13.5	0.2
9	9.06	8.83	8.61	8.41	8.22	0.10	9	13.2	12.9	12.6	12.3	12.0	0.1
10	8.04	7.87	7.70	7.54	7.38	0.08	10	11.7	11.5	11.2	11.0	10.7+	0.1
11	7.23	7.09	6.95	6.81	6.67	0.07	11	10.5+	10.3	10.1	9.9	9.7	0.1
12	6.54	6.42	6.30	6.18	6.07	0.06	12	9.52	9.34	9.17	9.00	8.83	0.08
13	5.96	5.86	5.76	5.66	5.56	0.05	13	8.67	8.51	8.36	8.22	8.09	0.07
14	5.47	5.38	5.29	5.21	5.13	0.04	14	7.96	7.83	7.71	7.59	7.47	0.06
15	5.05	4.97	4.90	4.83	4.76	0.04	15	7.35	7.24	7.13	7.02	6.92	0.05
16	4.69	4.62	4.56	4.50	4.44	0.03	16	6.82	6.72	6.63	6.54	6.45	0.05
17	4.38	4.32	4.26	4.20	4.15	0.03	17	6.36	6.28	6.20	6.12	6.04	0.04
18	4.10	4.05	4.00	3.95	3.90	0.02+	18	5.96	5.89	5.82	5.75	5.68	0.04
19	3.85	3.80	3.76	3.71	3.67	0.02	19	5.61	5.54	5.48	5.42	5.36	0.03
20	3.63	3.59	3.55	3.51	3.47	0.02	20	5.30	5.24	5.18	5.12	5.07	0.03
21	3.43	3.40	3.36	3.32	3.29	0.02−	21	5.02	4.96	4.91	4.86	4.81	0.03
22	3.25	3.22	3.18	3.15	3.12	0.02−	22	4.76	4.71	4.66	4.61	4.56	0.03
23	3.09	3.06	3.03	3.00	2.97	0.02−	23	4.52	4.47	4.42	4.38	4.34	0.02
24	2.94	2.91	2.88	2.86	2.83	0.01+	24	4.30	4.26	4.22	4.18	4.14	0.02
25	2.80	2.78−	2.75	2.73−	2.70	0.01+	25	4.10	4.06	4.02	3.98	3.94	0.02
26	2.68−	2.65	2.63	2.61−	2.58	0.01+	26	3.91	3.87	3.83	3.79	3.76	0.02
27	2.56	2.54	2.51−	2.50−	2.47+	0.01	27	3.73	3.69	3.66	3.63	3.60	0.02
28	2.45	2.43	2.41	2.39	2.37+	0.01	28	3.57	3.54	3.51	3.48	3.45	0.01+
29	2.36−	2.34	2.32	2.30	2.28	0.01	29	3.43	3.40	3.38	3.35	3.33	0.01
30	2.27−	2.25−	2.23	2.21	2.20−	0.01	30	3.30	3.28	3.25	3.22	3.20	0.01
31	2.18	2.17−	2.15	2.13	2.12−	0.01	31	3.18	3.15	3.13	3.11	3.09	0.01
32	2.10	2.09	2.07	2.06	2.04		32	3.07	3.04	3.02	3.00	2.98	0.01
33	2.03	2.02	2.00	1.99	1.97		33	2.96	2.94	2.92	2.90	2.88	0.01
34	1.96	1.95	1.94	1.92	1.91		34	2.86	2.84	2.82	2.80	2.78	0.01
35	1.90	1.88	1.87	1.86	1.85		35	2.77	2.75	2.73	2.71	2.70	
36	1.84	1.82	1.81	1.80	1.79		36	2.68	2.66	2.64	2.63	2.61	

Column D contains the values for increments or decrements corresponding to an increase of the readings by 0. mm. on the colorimeter scale, for use in interpolation.

The values are calculated for a dilution of 10 (as in inorganic P filtrate). When the dilution is 20, 30, or 40 (a in "total inorganic P" for phosphatase), these figures are to be multiplied by 2, 3, or 4 respectively.

When 2 cc. aliquots are used, deduct 3 per cent from the value for the 4 cc. aliquot and multiply by 2.

2. *Check.*
   a. Place 6 cc. of water in one large test tube and 6 cc. of 10% trichloroacetic acid in another.
   b. Add 2 cc. of the acid-molybdate mixture to each tube and mix.
   c. Add 2 cc. of dilute stannous chloride solution to each tube and mix.
   d. If the reagents are of suitable quality and properly prepared, the solutions in the two test tubes will be colorless or at the most tinged faintly green or blue.

E. *Permanent Solutions.*

1. *Trichloroacetic Acid*—10%.
2. *Sodium Molybdate Solution*—7.5%.
3. *Sulfuric Acid*—10 N.
   a. Add 290 cc. of conc. sulfuric acid (sp. gr. 1.84) to about 600 cc. of water in a liter volumetric flask, cool, and dilute to volume with water.
   b. Standardize and dilute if necessary.
   c. Keep in the refrigerator.
4. *Stannous Chloride Solution*—60%.
   a. Dissolve 15 gm. of stannous chlorid (Baker Analyzed) in about 15 cc. o conc. HCl (app. 36%) in a 25 cc. volu metric flask.
   b. Dilute to volume with conc. HCl.
   c. Keep in the refrigerator in a glass-stop pered bottle.
   d. Make fresh monthly.
5. *Standard Phosphate Solutions.*
   a. *Stock standard phosphate solution.*
      1) Dissolve 73.3 mg. of dried c.p. po tassium acid phosphate (KH_2PO_4) i about 200 cc. of water in a 250 cc volumetric flask.
      2) Add 1 cc. of conc. sulfuric acid an dilute to volume with water.
      3) Add a few drops of chloroform a a preservative.
   b. *Dilute standard phosphate solution* (6 cc

= 0.02 mg. P).

1) Pipette 5 cc. of the stock standard phosphate solution into a 100 cc. volumetric flask and dilute to volume with water.
2) Preserve with a few drops of chloroform.

F. ***Interpretation of Blood Inorganic Phosphorus Findings*** (See Table 85).

1. *Normal Values:*
   a. Adults: 2.5—4 mg. per cent.
   b. Children: 4.5—5.5 mg. per cent.
   c. Infants: 5.5—6.5 mg. per cent.
2. *Increased in:*
   Nephritis
   Pyloric obstruction
   After pituitrin administration
   Hypoparathyroidism
   Starvation
   During healing of fractures
   Renal rickets
   Hyperinsulinism
   Hypervitaminosis (esp. D)
   Uremia
3. *Decreased in:*
   Rickets
   Osteomalacia
   Neurofibromatosis
   Idiopathic steatorrhea
   Myxedema
   Hereditary periodic paralysis
   Lobar pneumonia
   Sprue
   Hyperparathyroidism
   After insulin or adrenalin administration
   During ether, chloroform, and ethylene anesthesia

XII. Inorganic Phosphorus (Gomori's Photoelectric Colorimeter Method).

A. ***Principle:*** Same as Bodansky's method since this is a modification of his method.

B. ***General Considerations.*** Same as Bodansky's method.

C. ***Method.***

1. Place 4 cc. of 5% trichloroacetic acid in a test tube and add dropwise 1 cc. of serum while shaking the tube vigorously.
2. Stopper, mix well, and let stand for 10 minutes.
3. Filter through 7 cm. Whatman No. 40 filter paper.
4. Pipette 1 cc. of filtrate into a colorimeter tube and add 6 cc. of water.
5. Place 7 cc. of water in a colorimeter tube for the blank.
6. To each tube add 2 cc. of acid-molybdate mixture and mix.
7. Add 1 cc. of Elon solution, mix, and read between 45 and 90 minutes.
8. Set the galvanometer at 100 with the blank using filter No. 540 and then read the unknown.
9. Obtain the mg. per cent of phosphorus from the table of values.
10. Periodically it is advisable to check the reagents and technique by setting up a standard consisting of 1 cc. of dilute working standard (1 cc. = 0.01 mg. P.) and 6 cc. of water at the same time as setting up the unknown.
11. *Calibration of Standard Curve.*
    a. Make a dilute working standard phosphorus solution (1 cc. = 0.01 mg. P.).
    b. Pipette the following amounts of the dilute working standard solution and water into a series of colorimeter tubes.

cc. of dilute working standard	cc. of water	mg.% of phosphorus in serum
0.5	6.5	2.5
1.0	6.0	5.0
2.0	5.0	10.0
3.0	4.0	15.0
4.0	3.0	20.0
0.0	7.0	Blank

    c. Develop the color as described in the method.
    d. Set the galvanometer at 100 with the blank using filter No. 540.
    e. Repeat tests several times using different dilute standard solutions and then repeat using a new stock standard solution.
    f. Average the galvanometer readings for each concentration and when plotted on semilogarithmic graph paper they should make a straight line.
    g. Make a table of values according to mg. per cent of phosphorus in the blood for each galvanometer division.

D. ***Solutions.***

1. *Trichloroacetic Acid*—5%.
2. *Sodium Molybdate Solution*—5% (keeps 1 month).
3. *Sulfuric Acid*—10 N (see solutions under Bodansky's method).
4. *Acid-Molybdate Mixture* (prepare daily).
   2 parts 5% sodium molybdate
   1 part 10 N sulfuric acid
   1 part water
5. *Sodium Bisulfite Solution* — 3% (prepare daily).
6. *Elon Solution* (prepare daily).
   a. Place 1 gm. Elon (Eastman Kodak) in a

Table 85. Diseases with Disturbed Calcium and Phosphorus Metabolism

Pathological Condition	Serum			Urine		Feces
	Ca	P	Alkaline phosphatase	Ca	P	Ca
Alkalosis with tetany (excessive vomiting, hyperventilation, and bicarbonate therapy)	N*	N	N	N	N	N
Carcinoma (metastatic to bone)	N to Inc.	N	N to Inc.**	Inc.	Inc.	
Hyperparathyroidism	Inc.	Dec.	N to Inc.	Inc.	Inc.	N
Hypoparathyroidism	Dec.	Inc.	N	Dec.	Dec.	N
Hyperthyroidism	N	N	N to Inc.	Inc.	Inc.	Inc.
Hyperproteinemia***	Inc.	N				
Hypoproteinemia***	Dec.	N				
Hypervitaminosis (vit. D therapy)	Inc.	Inc.	N	Inc.	Inc.	
Idiopathic steatorrhea (tropical and nontropical sprue, celiac disease)	Dec. to N	Dec. to N	N to Inc.	Dec.	Dec.	Inc.
Multiple myeloma	Inc.	N	N	N to Inc.	N to Inc.	
Neurofibromatosis	N to Inc.	Dec. to N	N to Inc.			
Obstructive jaundice	N	N	Inc.			
Osteogenic sarcoma	N	N	N to Inc.	N	N	
Osteomalacia	Dec. to N	Dec.	Inc.	Dec.	Dec.	Dec.
Paget's disease (Osteitis deformans)	N	N	Inc.	N to Inc.	N	
Renal rickets	Dec. to N	Inc.	Inc.	Dec.	Dec.	
Rickets	N	Dec. to N	Inc.	Dec.	Dec.	
Uremia	Dec.	Inc.	N	Dec.	Dec.	Inc.

N = normal. Inc. = increased. Dec. = decreased.
*Ionized calcium decreased. **Acid phosphatase also increased in carcinoma of the prostate.
***Calcium metabolism normal.

100 cc. volumetric flask.

b. Dissolve in and dilute to volume with 3% sodium bisulfite solution.

7. *Standard Phosphorus Solutions.*
   - a. *Stock standard solution* (1 cc. = 1 mg. P).
     - 1) Place 4.3904 gm. of dried c.p. potassium acid phosphate (KH_2PO_4) in a liter volumetric flask and dissolve in about 700 cc. of water.
     - 2) Dilute to volume with water and add a few drops of chloroform as a preservative.
     - 3) Keeps indefinitely.
   - b. *Dilute standard solution* (1 cc. = 0.1 mg. P).
     - 1) Pipette 10 cc. of the stock standard solution into a 100 cc. volumetric flask and dilute to volume with water.
     - 2) Add a few drops of chloroform as a preservative.
   - c. *Dilute working standard solution* (1 cc. = 0.01 mg. P).
     - 1) Pipette 5 cc. of dilute standard solution (1 cc. = 0.1 mg. P) into a 50 cc. volumetric flask and dilute to volume with water.
     - 2) Must be prepared on day to be used.

E. ***Interpretation***—same as in Bodansky's method.

Blood Enzymes

I. Alkaline Phosphatase (Bodansky's Colorimeter Method).

A. ***Principle:*** The difference between the total inorganic phosphorus, after incubation of the serum with a sodium glycerophosphate substrate, and the serum inorganic phosphorus without such incubation is used as a measure of phosphatase activity. The Bodansky unit of phosphatase activity is defined as that amount of activity which will liberate 1 mg. of inorganic phosphorus per 100 cc. of serum from a sodium glycerophosphate substrate solution (pH 8.6) during the first hour of incubation at 37°C.

General Considerations.

Analysis may be delayed for several hours if the serum is kept in the refrigerator. If a longer delay is necessary, add a small drop of toluene.

After serum has remained in the refrigerator for 24 to 48 hours, the phosphatase is about 10 to 15% higher than originally. At room temperature (as in mailed specimens) the results are subject to an error of 20%.

Sources of error.

a. Incorrect temperature of water bath which should not vary more than ±1°C.
b. Incorrect timing of incubation.
c. Substrate too old or incorrect pH.
d. Same errors as for phosphorus determination.

Method.

1. Allow substrate (sodium glycerophosphate) to come to room temperature, measure 10 cc. into a test tube (18 or 20 × 150 mm.) avoiding aeration and close tube immediately with a rubber stopper.
2. Place in a water bath at 37°C. for 5 minutes, add 1 cc. of serum with the tip of the pipette about 1 cm. above the surface of the liquid, and again stopper immediately.
3. Tap the test tube to impart rotary motion so as to mix the contents and to wash down any serum on the side of the tube.
4. Replace in the water bath for exactly 1 hour and keep at exactly 37°C. (±1°) because this is the temperature at which phosphatase liberates inorganic phosphorus from the substrate.
5. Remove the test tube from water bath. Cool immediately in ice water for about 2 minutes.
6. Add 9 cc. of 10% trichloroacetic acid, mix, and after 20 minutes filter through a 9 cm. Whatman No. 40 filter paper (Filtrate A). When a high phosphatase activity is expected, a higher dilution may be made by the addition of a larger volume of 10% trichloroacetic acid.
7. Filtrate A = total inorganic phosphorus filtrate (serum inorganic phosphorus plus inorganic phosphorus liberated from the substrate by the serum phosphatase).
8. Filtrate B = serum inorganic phosphorus filtrate. This is necessary so that the serum inorganic phosphorus can be subtracted from the total inorganic phosphorus to find the phosphatase activity of the serum.
   a. To make filtrate B, pipette 9 cc. of 10% trichloroacetic acid into a 15 cc. test tube, add 1 cc. of serum, stopper, mix well, and let stand 20 minutes.
   b. Filter in the same manner as Filtrate A.
9. The filtrates may be saved in the refrigerator until analyzed for inorganic phosphorus; analysis performed immediately and after several days yield excellent checks.
10. Place 6 cc. of the dilute standard phosphate solution (6 cc. = 0.02 mg. P) in each of two large test tubes.
11. Place 2, 4, and 6 cc. portions of Filtrate A in each of three large test tubes and make up to 6 cc. with water.
12. Place 6 cc. of Filtrate B in a large test tube.
13. Add the 2 following reagents consecutively to each tube as follows:
    a. Two cc. of acid-molybdate mixture (freshly made and checked) and mix contents by tapping tube.
    b. Blow in 2 cc. of dilute stannous chloride reagent (freshly made and checked), mixing during the addition.
14. The color develops rapidly and comparison may be made immediately or any time within 2 hours.
15. Check standards against each other before making comparisons of unknowns.
16. *Calculation:*
    a. The value for the colorimeter reading of Filtrate B, "serum inorganic P," in mg. per 100 cc. is found in Table 84 under the division corresponding to the volume used.
    b. The value for the colorimeter reading of Filtrate A, "total inorganic P," in mg. per 100 cc. is found in Table 84 under the division corresponding to the volume used, but is multiplied by 2 because the dilution was 20 instead of 10. When dilutions of 30 or 40 are employed, the value is multiplied by 3 or 4 respectively.
    c. The units of phosphatase activity per 100 cc. equals the value for Filtrate A minus the value for Filtrate B.

D. *Solutions.*

1. *Buffered Substrate.*
   a. Into a 100 cc. volumetric flask introduce successively 3 cc. of petroleum ether

(b.p. 20 to 40°C.), about 80 cc. of distilled water, 0.5 gm. of sodium beta-glycerophosphate [$NaC_3H_5(OH)_2PO_4$, Eastman], 0.424 gm. of sodium diethylbarbiturate (Merck), and water to volume (read at interface between substrate and petroleum ether).
   - b. Empty into a 200 cc. glass-stoppered Pyrex bottle containing about 1 inch of petroleum ether.
   - c. Keeps 2 months in the refrigerator.
   - d. When multiples of 100 cc. are made, it is advisable to distribute the substrate into bottles in 100 cc. portions.
   - e. If sodium beta-glycerophosphate cannot be obtained, 0.708 gm. of sodium alpha-glycerophosphate [$NaC_3H_5(OH)_2PO_4 \cdot 5H_2O$] may be substituted if the prepared substrate is allowed to stand 2 weeks in the refrigerator before using.

2. For other solutions—see inorganic phosphorus determination, page 308.

E. *Interpretation of Alkaline Phosphatase Findings* (See Table 85).

1. *Normal Values:*
   - a. Adults: 1.5—4 units.
   - b. Children: 5—12 units.
2. High serum phosphatase is a manifestation of processes that cause rapid growth of bone in the normal young, of new bone (repair) and of calcified and decalcified pathologic bone.
3. *Increased in:*
   Diseases of the bone producing increased osteoblastic activity; such as, osteomalacia, osteoblastic sarcoma, or metastatic carcinoma (breast, lung, and prostate).
   Osteitis deformans (Paget's disease)
   Neurofibromatosis
   Myositis ossificans
   Rickets
   Hyperparathyroidism
   Renal rickets
   Hodgkin's disease with bone involvement
   Boeck's sarcoid
   Multiple myeloma
   Hyperthyroidism
   Obstructive jaundice
   Infectious hepatitis
   Occlusion of pancreatic duct
   Idiopathic steatorrhea
4. *Decreased in:*
   Hypothyroidism
   Scurvy
   Celiac disease
   Severe chronic nephritis
   Osteolytic sarcoma

II. Alkaline Phosphatase (Gomori's Ph
electric Colorimeter Method).

A. *Principle:* Same as in Bodansky's met

B. *General Considerations:* Same as in Bo
sky's method.

C. *Method.*

1. Allow substrate (sodium glycerophosph
to come to room temperature, measure 10
into a large test tube avoiding aeration
close with a rubber stopper.
2. Place in a 37°C. water bath for 5 minu
then add 1 cc. of serum with the tip of
pipette about 1 cm. above the surface
the liquid and again stopper immediat
3. Tap the test tube to impart rotary mot
so as to mix the contents and to wash do
any serum on the side of the tube.
4. Return to the water bath for exactly
hour and keep at exactly 37°C.
5. Immediately add 5 cc. of 20% trichlo
acetic acid and mix.
6. After 10 minutes filter through Whatm
No. 40 filter paper (Filtrate A).
7. Determine the total phosphorus in Filtr
A by Gomori's photoelectric colorime
method for inorganic phosphorus using 2
of acid-molybdate mixture, 2 cc. of filtra
and 5 cc. of water in a colorimeter tube.
8. At the same time determine the inorga
phosphorus in a Filtrate B made as
scribed in Gomori's photoelectric colorime
method.
9. Obtain the mg. per cent of phosphorus
the galvanometer reading of Filtrate A fr
the table of values made for the phospho
method.
10. To obtain the values for total phosphor
multiply by 8/5 because 2 cc. of a 1-
dilution was used.
11. Obtain mg. per cent of inorganic phospho
for Filtrate B from same table of valu
12. Phosphatase in Bodansky units per 100
of serum = total phosphorus (Filtrate
— inorganic phosphorus (Filtrate B).

D. *Solutions.*

1. *Trichloroacetic Acid*—20%
2. *Buffered Substrate*—see direction in Boda
sky's method of alkaline phosphatase.
3. For other solutions see Gomori's photoelectr
colorimeter method for inorganic phosph
rus.

E. *Interpretation*—see Bodansky's method f
alkaline phosphatase.

III. Acid Phosphatase (Gutman and Gutman's Method).

A. ***Principle:*** The difference between phenol produced after incubation of serum with a monophenylphosphate substrate and that produced without incubation is used as a measure of acid phosphatase activity. The King and Armstrong unit of alkaline phosphatase activity is used and defined for acid phosphatase as that amount of activity which will liberate 1 mg. of phenol from the buffered monophenylphosphate at pH 4.9 during 1 hour of incubation at 37°C.

B. ***General Considerations.***

1. The monophenylphosphate substrate must have a pH of 4.9.
2. When 15 minutes incubation still gives high readings, the serum must be diluted with 0.85% NaCl solution and the test repeated using controls with the diluted serum.
3. A blank containing water instead of serum should be incubated along with the sample containing the phosphatase because of the instability of the monophenylphosphate in an acid medium at 37°C.
4. *Sources of error.*
   a. Incorrect temperature of water bath.
   b. Incorrect timing of incubation.
   c. Incorrect pH of substrate.

C. ***Colorimetric Method:***

1. Pipette 10 cc. of freshly prepared buffered substrate into each of 3 test tubes.
2. Place the tubes in a 37°C. water bath for 5 minutes.
3. Pipette exactly 0.5 cc. of serum into each of 2 tubes and 0.5 cc. of water into the other tube (blank), stopper, then mix by tapping the tubes, and incubate at 37°C. for exactly 3 hours. (If acid phosphatase activity is high, incubate for a shorter period.)
4. Remove tubes from water bath and immediately add 4.5 cc. of dilute phenol reagent to each.
5. Mix by inverting and filter.
6. *Control tube.*
   a. Pipette 10 cc. of the buffered substrate into a test tube and add 0.5 cc. of serum.
   b. Immediately add 4.5 cc. of dilute phenol reagent, mix, and filter.
7. Pipette 6 cc. of each filtrate (3 incubated and 1 control) into test tubes.
8. *Standard.*
   a. Prepare just before ready to use.
   b. Pipette 1 cc. of dilute standard phenol solution (1 cc. = 0.1 mg.) into a test tube.
   c. Add 6 cc. of water and 3 cc. of dilute phenol reagent.
   d. Add 2.5 cc. of 20% sodium carbonate solution and mix.
9. To each of the other 4 tubes, add 1.5 cc. of 20% sodium carbonate solution and mix.
10. Place all the tubes in a 37°C. water bath for 5 minutes to develop the color.
11. Remove from water bath and cool to room temperature for 20 minutes.
12. Compare in the colorimeter with the standard set at 10.
13. If the results are abnormally high, repeat the test decreasing the time of incubation.
14. *Calculation for serum, blank, and control:*
    a. $\frac{RS}{RU} \times 0.1 \times \frac{7.5}{12.5} \times \frac{15}{6} \times \frac{100}{0.5} = \frac{300}{RU}$ = mg.% of phenol.
    b. Units of phosphatase in 100 cc. of serum equals (mg. of phenol in incubated serum) minus (mg. of phenol formed in blank plus mg. of phenol in serum control) divided by hours of incubation.

D. ***Photoelectric Colorimeter Method.***

1. Follow the colorimetric method through step 6.
2. Pipette 5 cc. of each filtrate (3 incubated and 1 control) into colorimeter tubes and add 5 cc. of water to each tube.
3. Add 2.5 cc. of a 20% solution of sodium carbonate to all of the tubes, shake well, and place in a 37°C. water bath for 5 minutes.
4. Remove the tubes and allow to stand at room temperature for 20 minutes.
5. Using filter No. 420, set the galvanometer at 100 with the blank (the tube labeled blank in the colorimetric method) and then read the unknown and control.
6. Obtain the mg. per cent of phenol for each tube from the table of values.
7. Units of phosphatase in 100 cc. of serum equals (mg. of phenol in incubated serum) minus (mg. of phenol in serum control) divided by hours of incubation.
8. *Calibration of Standard Curve.*
   a. Prepare the following dilutions from the dilute standard phenol solution (1 cc. = 0.1 mg.).

cc. of dilute standard phenol solution	Dilute with water to	Dilution
4	200 cc.	1—50
5	100 cc.	1—20
5	50 cc.	1—10
10	50 cc.	1—5

b. Set up 7 colorimeter tubes as follows:

Tube	cc. of above dilutions	cc. of water	mg.% of phenol in serum
1	5 of 1—50	2	6
2	4 of 1—20	3	12
3	4 of 1—10	3	24
4	6 of 1—10	1	36
5	4 of 1—5	3	48
6	5 of 1—5	2	60
7	none	7	Blank

c. Add 3 cc. of the dilute phenol reagent to each tube.

d. Add 2.5 cc. of a 20% solution of sodium carbonate to each tube, mix well, and place in a 37°C. water bath for 5 minutes.

e. Remove the tubes and allow to stand at room temperature for 20 minutes.

f. Using filter No. 420, set galvanometer at 100 with tube 7 and read the other tubes.

g. Repeat several times with the same dilutions and then repeat using a new dilute standard phenol solution.

h. Average the galvanometer readings for each tube and plot the results on semi-logarithmic paper. A line drawn through the points should be a straight line.

i. Prepare a table of values for the mg. per cent of phenol for each division on the galvanometer.

E. *Solutions.*

1. *Buffered Substrate* (pH 4.9).
   - a. *Solution A.*
     - 1) Dissolve 1.09 gm. of disodiumphenylphosphate ($Na_2C_6H_5PO_4$) in 500 cc. of water.
     - 2) This is 0.005 M monophenolphosphate.
     - 3) Keeps 2 months in the refrigerator.
   - b. *Solution B.*
     - 1) Dissolve 42 gm. crystalline citric acid in about 500 cc. of water in a liter volumetric flask.
     - 2) Add 376 cc. of 1 N NaOH and make up to volume with water.
     - 3) Adjust pH to 4.9 with NaOH or HCl as needed, testing with a pH meter. Keep in the refrigerator.
   - c. *Buffered substrate* (made day of test).
     - 1) Mix equal parts of Solutions A and B as needed.
     - 2) Allow to come to room temperature before pipetting for test.
2. *Phenol Reagent of Folin and Ciocalteu.*
   - a. Place 100 gm. of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 25 gm. sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) together with 700 cc. of water into a 2 liter Florence flask.
   - b. Add 50 cc. of 85% phosphoric acid a 100 cc. of conc. HCl.
   - c. Connect with a reflux condenser b means of a cork or rubber stopp wrapped in tin foil and boil gently f 10 hours.
   - d. At the end of the boiling period, ad 150 gm. of lithium sulfate, 50 cc. o water, and a few drops of liquid bromine
   - e. Boil the mixture without the condense for about 15 minutes to remove the excess bromine.
   - f. Cool, dilute to 1 liter with water, and filter.
   - g. The finished reagent should have no greenish color.
   - h. Keep in a well stoppered brown bottle and dilute 1 part of reagent with 2 parts of water before using.
3. *Sodium Carbonate Solution—20%.*
4. *Stock Standard Phenol Solution.*
   - a. Weigh 1 gm. of crystalline phenol and place in a liter volumetric flask.
   - b. Dissolve in about 700 cc. of 0.1 N HCl and dilute to volume with 0.1 N HCl.
   - c. Solution keeps indefinitely.
   - d. *Standardization.*
     - 1) Transfer 25 cc. of this solution to a 250 cc. flask, add 50 cc. of a 0.1 N NaOH solution, and heat to 65°C.
     - 2) Add 25 cc. of a 0.1 N iodine solution, stopper flask, and let stand at room temperature for 30 to 40 minutes.
     - 3) Add 5 cc. conc. HCl and titrate the excess of iodine with 0.1 N sodium thiosulfate solution.
     - 4) When the iodine color has faded to a pale yellow, add 1 cc. of 1% soluble starch and titrate until the blue color of the starch-iodine compound has entirely disappeared.
     - 5) Each cc. of 0.1 N iodine solution used up (25 cc. — cc. of thiosulfate used) corresponds to 1.567 mg. of phenol.
     - 6) For directions to prepare 0.1 N sodium thiosulfate solution and 0.1 N iodine solution, see Section on Solutions, page 358.
5. *Dilute Standard Phenol Solution* (1 cc. = 0.1 mg.).
   - a. On the basis of the standardization, dilute the stock standard phenol solution so that 100 cc. contains 10 mg. of phenol.
   - b. This dilute solution remains stable for at least 3 months in the refrigerator.

b. If too long a time is taken for the titration, too high values will be obtained.

c. Over titration of blank.

C. *Method.*

1. Pipette 0.2 cc. of plasma into a 15 cc. conical centrifuge tube and add 0.2 cc. of water and 0.4 cc. of fresh 5% metaphosphoric acid solution.
2. Mix thoroughly and centrifuge to remove the precipitated protein.
3. Make a fresh 1-20 dilution of the stock dye solution with water.
4. Fill the microburette (Fig. 30) by placing the dye solution in a clean test tube which is held nearly horizontal when slipped over the curved end of the burette. Turn the screw to the right until a small drop of mercury is expelled into the dye solution and then fill the burette to the desired point by turning the screw in the reverse direction.

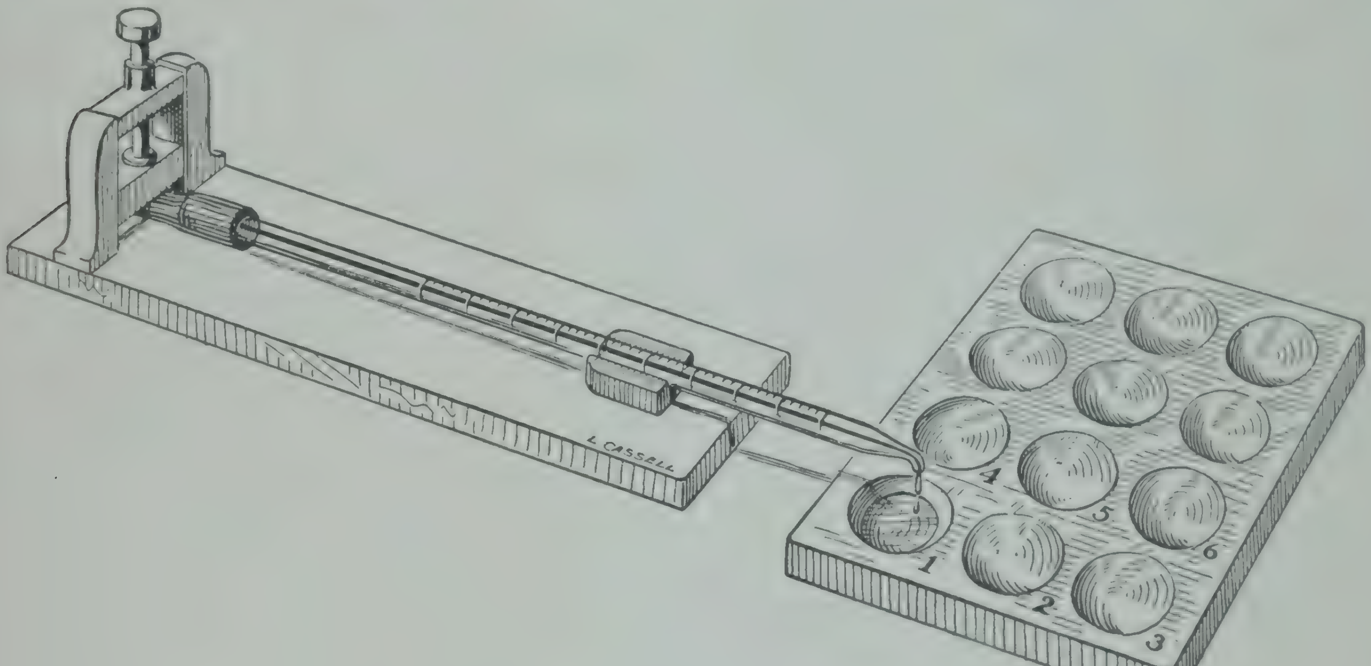


FIG. 30. APPARATUS FOR THE TITRATION OF ASCORBIC ACID.

5. Place 0.1 cc. of 5% metaphosphoric acid and 0.1 cc. of water in depressions 4 and 6 of the titration tile (see Fig. 30).
6. Place 0.2 cc. of water in depression 2. This is used as a color control.
7. Into depressions 1 and 3, pipette 0.2 cc. of the sample of deproteinized plasma.
8. Read the microburette and titrate the blank in depression 4 until the first discernible trace of color (faint pink) is obtained after mixing with a stirring rod having a fine point.
9. Record burette reading.
10. Titrate the deproteinized plasma in depression 1 until the color matches that of the blank just titrated and record the burette reading.
11. Titrate the duplicates in depressions 6 and 3.
12. Subtract the average of the readings of the blanks (4 and 6) from the average of the readings of the unknowns (1 and 3).
13. *Calculation:*

$$\text{cc. of dye (cc. unknown — cc. blank)} \times \frac{S}{20} \times \frac{100}{0.05} = \text{mg. \% ascorbic acid.}$$

S = mg. ascorbic acid equivalent to 1 cc. of stock dye (see standardization of dye solution below).

D. *Solutions.*

1. *Metaphosphoric Acid Solution—5%.*
   a. Grind up about one-half inch of a stick of metaphosphoric acid (HPO_3) in a mortar.
   b. Weigh out 1 gm. of this powder and place in a small Erlenmeyer flask.
   c. Add 19 cc. of water, shake until dissolved, and filter.
   d. Can be used the second day if kept in the refrigerator.
2. *Dilute Dye Solution for Test* (1-20 dilution).
   a. Allow stock dye solution to come to room temperature, then place 0.5 cc. in a 10 cc. graduated glass-stoppered cylinder.
   b. Dilute to 10 cc. with water.
   c. A solution having any pink tinge to it must be discarded.
   d. Can be used the second day if kept in the refrigerator.

E. *Preparation and Standardization of Stock Dye Solution.*

1. *Stock Dye Solution.*

a. Weigh 50 mg. of dye (sodium 2, 6-dichlorobenzenoneindophenol, Eastman) and transfer to a small folded filter paper in a funnel.
b. Place funnel in a 50 cc. volumetric flask and slowly pour 40 cc. of boiling water through the filter paper.
c. When flask of dye solution has cooled to room temperature, dilute to volume with water.
d. Keeps 1 month in the refrigerator.

2. *Standard Ascorbic Acid Solutions.*
   a. *Stock standard solution* (1 cc. = 0.5 mg.).
      1) Weigh exactly 50 mg. of ascorbic acid and place in a 100 cc. volumetric flask.
      2) Dilute to volume with a freshly prepared 2.5% metaphosphoric acid solution (6.25 gm. diluted to 250 cc. with water).
   b. *Dilute standard solution* (1 cc. = 0.05 mg.).
      1) Pipette 10 cc. of the stock standard ascorbic acid solution into a 100 cc. volumetric flask.
      2) Dilute to volume with the freshly prepared 2.5% metaphosphoric acid solution.
3. *Standardization of the Dye Solution.*
   a. Prepare a dilute dye solution (1-10 dilution) by pipetting 5 cc. of the stock dye solution into a 50 cc. volumetric flask and diluting to volume with water.
   b. Fill a 5 cc. burette graduated to 0.01 cc. with this dilute dye solution.
   c. In one test tube place 2 cc. of the dilute standard ascorbic acid solution; in another tube place 2 cc. of 2.5% metaphosphoric acid solution (blank).
   d. Titrate each with the dye solution to the first faint pink color.
   e. The amount of dye used in titrating the blank is subtracted from that used in titrating the standard ascorbic acid solution.
   f. *Example calculation:*
      1) 2.27 cc. of dilute dye solution was used in titrating the dilute standard solution.
      2) 0.10 cc. of dilute dye solution was used in titrating the blank.
      3) 2.27 cc. — 0.10 cc. = 2.17 cc. of dilute dye solution which is equivalent to 2 × 0.05 mg. of ascorbic acid.

$$\text{Each cc. of stock dye} = \frac{2 \times 0.05}{2.17} \times 10 = 0.460 \text{ mg. of ascorbic acid.}$$

F. *Interpretation of Blood Ascorbic A* *Findings.*

1. *Normal Values:* above 0.6 mg. per cent.
2. *Decreased in:*
   Scurvy
   Vitamin C (ascorbic acid) deficiency

II. Urine Ascorbic Acid (Farmer and Al Method).

A. *Principle:* Same as in the blood ascorbic a method.

B. *General Considerations.*

1. Collect a 24 hour specimen of urine in a h gallon bottle containing 25 gm. of metaph phoric acid.
2. Keep bottle stoppered tightly and in t refrigerator during collection period.

C. *Method.*

1. Measure volume of urine.
2. Dilute urine 1-2 or 1-5 with 2.5% metapho phoric acid according to color of specime It should be diluted until color is lig enough that it will not interfere with recog nition of the pink end-point in the titratio
3. Place 2 cc. of the properly diluted urine a round bottomed test tube.
4. Using a 5 cc. burette, titrate with a 1-1 dilution of stock dye solution (sodium 2, dichlorobenzenoneindophenol) until the firs light pink color persists for 30 seconds.
5. A 2 cc. blank of 2.5% metaphosphoric aci is titrated to the same end-point.
6. *Calculation:*

$$\frac{(\text{cc. dye} - \text{cc. blank})}{2} \times \frac{S}{10}$$

× urine dilution × cc. 24 hr. urine = mg. of ascorbic acid per 24 hours.

S = mg. ascorbic acid equivalent to 1 cc. of stoc dye (see standardization of dye solution).

D. *Solutions.*

1. See blood ascorbic acid method.
2. Dye is a 1-10 dilution of the stock solution.

E. *Interpretation of Urine Ascorbic Acid Findings.*

1. Normal value is around 20 mg. per 24 hours on a fairly adequate diet.
2. With diets richer in ascorbic acid there will be a greater excretion in the urine.
3. Renal threshold is 0.8—1.4 mg. per 100 cc. of plasma.
4. Increased after ether anesthesia.

III. Vitamin A and Carotene in Plasma (Kimble's Method Modified by Friedemann and Miller).

A. *Principle:* Carotene is determined in a petroleum ether extract of plasma previously treated with alcohol. The yellow color is determined with a photoelectric colorimeter. The vitamin A contained in the evaporated extract upon the addition of antimony chloride produces a blue color which is measured with the photoelectric colorimeter. This blue color is too pale to measure in a visual colorimeter.

General Considerations.

This method is only applicable to colorimeters which have galvanometers. It can not be used for instruments using potentiometric methods to determine the degree of transmission.

Glassware which has previously contained antimony chloride must be cleaned with hydrochloric acid.

Antimony chloride is very corrosive and great care should be taken to prevent its touching the photoelectric colorimeter or the skin.

Test tubes used for high speed centrifuging should be tested for endurance before using in the test.

Chloroform destroys vitamin A; after it is added, the test must be completed immediately.

The results are of no clinical value if the patient has taken any vitamin A in the preceding month, unless the value is below normal.

Method.

Pipette 5 cc. of plasma into a 50 cc. centrifuge tube.

Quickly add 5 cc. of the alcohol-caprylic mixture.

Mix and add 15 cc. of petroleum ether from a burette.

Stopper and shake vigorously for at least 3 minutes.

Centrifuge by turning centrifuge up to 3500 revolutions per minute and immediately back to zero.

Remove the top layer of petroleum ether with a capillary pipette and transfer to a second 50 cc. centrifuge tube.

Quickly add 10 cc. of 50% alcohol and shake 30 seconds.

Centrifuge for 5 minutes at 3500 revolutions per minute.

Remove 10 cc. of the top layer with a volumetric pipette and place in a colorimeter tube.

10. Immediately read the color of this tube with filter No. 440 using 10 cc. of petroleum ether to set the galvanometer at 100; this gives the carotene value.
11. Evaporate the petroleum ether in the colorimeter tube to dryness by blowing nitrogen (or air) into the tube. This evaporation may be speeded up by placing the tube in a beaker of hot water and must be complete because any moisture will cause the antimony chloride solution to become cloudy.
12. Using filter No. 620, set the galvanometer at 100 with a blank consisting of 1 cc. of chloroform and 9 cc. of 25% antimony chloride solution.
13. Add 1 cc. of chloroform to the evaporated sample and immediately place the tube in the machine; add 9 cc. of 25% antimony chloride solution and read at once as the resulting blue color is unstable.
14. Obtain the I. U. per cent of carotene and vitamin A from the table of values.
15. The I. U. per cent of vitamin A in the carotene present is obtained from a graph similar to Fig. 31. This amount is subtracted from the I. U. per cent of vitamin A to obtain the true value for vitamin A in the blood.

D. *Calibration of Standard Curve for Carotene.*

1. Obtain carotene (crystalline) in 100 mg. ampules from General Biochemical Co., Chagrin Falls, Ohio.
2. *Stock Standard Carotene Solution* (1 cc. = 333.3 International Units (I.U.) of carotene).
   a. Weigh exactly 50 mg. of carotene in a weighing flask or small beaker and wash into a 250 cc. volumetric flask with 25 cc. of chloroform.
   b. Dilute to volume with petroleum ether at 20°C.
3. Make 2 dilute standard carotene solutions, A and B.
   a. For *standard A* (1 cc. = 8.33 I.U. carotene), pipette 5 cc. of the stock standard carotene solution into a 200 cc. volumetric flask and dilute to volume with the alcohol-caprylic mixture.
   b. For *standard B* (1 cc. = 1.66 I. U. carotene), pipette 10 cc. of standard A into a 50 cc. volumetric flask and dilute to volume with the alcohol-caprylic mixture.

4. Pipette the following into 8 centrifuge tubes.

cc. of standard solution	cc. of alcohol-caprylic mixture	Equivalent to I.U. carotene in 100 cc. of blood
1 (B)	4	33.2
2 (B)	3	66.4
3 (B)	2	99.6
4 (B)	1	132.8
5 (B)	0	166.0
2 (A)	3	333.2
3 (A)	2	499.8
4 (A)	1	666.4

5. Add 5 cc. of water to each and then 15 cc. of petroleum ether and mix.
6. Continue as described in the method and read the carotene with filter No. 440, then continue the test as for vitamin A and read with filter No. 620. This will give the amount of substance in the carotene which will give a blue color in the test for vitamin A.
7. Make a sufficient number of determinations so that an average of the galvanometer readings with filter No. 440 will make a straight line when plotted on semilogarithmic graph paper.
8. Tabulate the International Units per cent of carotene for each division on the galvanometer.
9. Average the galvanometer readings for each tube with the 620 filter and obtain the equivalent I. U. % of vitamin A for each reading from the vitamin A table of values.
10. Plot on plain graph paper the I. U. % of vitamin A for each tube against the corresponding I. U. % of carotene, see Fig. 31.
11. The amount of vitamin A corresponding to the amount of carotene in a plasma determination must be subtracted from the vitamin A of that sample to obtain the true vitamin A content of the blood.

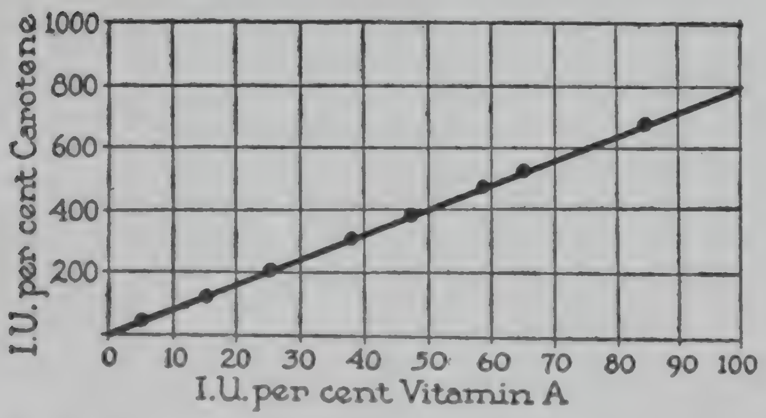


FIG. 31. Example of a chart to obtain the I. U. per cent of vitamin A in carotene which must be subtracted from the I. U. per cent of vitamin A to obtain the true vitamin A value.

E. ***Calibration of Standard Curve for V min A.***

1. Obtain vitamin A oil in 1 cc. ampules fr Distillation Products, Inc., Rochester, N. The vitamin should have an E. coeffic of 100 and contain 200,000 Internati Units (I. U.) per gram.
2. *Stock Standard Vitamin A Solution.*
   a. Weigh a 100 cc. volumetric flask on analytical balance.
   b. Take up as much of the vitamin A possible into a 1 cc. syringe and inj carefully into the flask.
   c. Reweigh the flask. The difference is weight of the vitamin A.
   d. Add 25 cc. of absolute alcohol and cc. of 10 N KOH.
   e. Incubate in a 50°C. water bath for minutes, cool, and dilute to volume 20°C. with absolute alcohol.
   f. Calculate the I. U. in each cc. of th stock solution according to the weig of the vitamin as obtained above; i.e., exactly 1 gram was used then 1 cc. = 2,000 units. This value will be used for example in making dilutions for the cal bration curve.
3. Make a *dilute standard vitamin A solutio* (1 cc. = 200 I. U.) by pipetting 10 cc. the stock standard solution into a 100 c volumetric flask and diluting to volume wi absolute alcohol.
4. From the dilute standard vitamin A solutio make standard solutions A and B.
   a. For *standard A* (1 cc. = 2 I. U.), p pette 5 cc. of the dilute standard solu tion into a 500 cc. volumetric flask an dilute to volume with absolute alcoho
   b. For *standard B* (1 cc. = 4 I. U.), p pette 5 cc. of the dilute standard solu tion into a 250 cc. volumetric flask an dilute to volume with absolute alcoho
5. Pipette the following into 8 extraction tube

cc. of standard solution	cc. of alcohol-caprylic mixture	Equivalent to I. U. of vit. A. in 100 cc. of bloo
1 (A)	4	40
2 (A)	3	80
3 (A)	2	120
4 (A)	1	160
5 (A)	0	200
3 (B)	2	240
4 (B)	1	320
5 (B)	0	400

6. Add 5 cc. of water and 15 cc. of petroleu ether to each tube and mix.
7. Continue with method above but do n read for carotene.

8. Make a sufficient number of determinations so that an average of the galvanometer readings for each tube will make a straight line when plotted on semilogarithmic graph paper.
9. Tabulate the International Units per cent of vitamin A for each division on the galvanometer.

. ***Solutions.***

Alcohol-Caprylic Mixture—Add 2.5 cc. of caprylic alcohol to 100 cc. of absolute alcohol.

Petroleum Ether having a boiling point of 63 to 70°C. (146-156°F.).

Antimony Chloride Solution—25%.

a. Pulverize 110 gm. of antimony chloride ($SbCl_3$) in a mortar.
b. Weigh a brown bottle which has been marked at 400 cc.
c. Transfer the antimony chloride to a stiff white paper and then to the weighed bottle until 100 gm. has been added.
d. Make up to volume with chloroform at room temperature.
e. Swirl the bottle to aid the solution of the antimony chloride.
f. Do not use until completely in solution; this usually requires about 12 hours.
g. If not clear, filter.

. ***Interpretation of Plasma Carotene and Vitamin A Findings.***

Carotene.

a. *Normal values:* 100—300 I. U. per cent.
b. *Increased in:*
   Carotenemia
   Severe liver damage
c. *Decreased in:*
   Obstructive jaundice

Vitamin A.

a. *Normal values:* 100—250 I. U. per cent.
b. *Increased in:*
   Renal insufficiency
c. *Decreased in:*
   Vitamin A deficiency
   Severe infections (esp. pneumonia)
   Severe liver damage
   Obstructive jaundice
   Infectious hepatitis
   Rheumatic fever
   Sprue

V. Lipochromic Index (Connor's Method).

Principle: Lipochromes are yellow pigments containing neutral fat and cholesterol, such as carotene, the pigments in butter, etc. They are bound to protein and set free by the precipitation of the protein with alcohol. The lipochromes are then soluble in petroleum ether.

B. *General Considerations.*

1. This method is only an approximate quantitative estimation.
2. Other blood pigments such as bilirubin, etc., are not soluble in petroleum ether.

C. *Method.*

1. Place 6 cc. of serum in a test tube, add 6 cc. of 95% alcohol, and mix.
2. After the mixture coagulates, add 8 cc. of petroleum ether, cork immediately, and shake vigorously for 1 minute.
3. When the precipitate has settled, transfer the ether to a colorimeter cup and compare with the standard set at 15 mm.
4. The standard is a 0.02% aqueous solution of potassium dichromate (equivalent to 0.1 mg. % of lipochromes).
5. *Calculation:*

$$\frac{RS}{RU} \times 0.1 \times \frac{8}{6} = \text{mg. \%}$$

D. *Interpretation of the Lipochromic Index.*

1. *Normal Values:* 0—0.1 mg. per cent.
2. *Increased in* carotenemia and sometimes in diabetes.

V. Pyruvic Acid (Friedemann and Haugen's Method).

A. *Principle:* When a solution of 2, 4-dinitrophenylhydrazine is added to a protein-free blood filtrate, the hydrazones of keto acids are formed (pyruvic acid is the principal keto acid in blood). These are first extracted with ethyl acetate and then with a sodium carbonate solution. A red color develops on the addition of an alkali and the amount of pyruvic acid is determined by means of a photoelectric colorimeter.

B. *General Considerations.*

1. All solutions must be pipetted *very carefully*.
2. Due to the volatility of the solvent, each determination must be completed as soon as possible.
3. If many determinations are made, the phenylhydrazine reagent is added to successive samples at definite intervals, i.e., every 30 seconds.
4. The results are of no clinical value if the patient has taken any vitamin B_1, in the preceding month, unless the pyruvic acid value is increased.

C. *Method.*

1. The patient should not eat for at least 4

hours and should rest for 30 minutes before the blood is drawn as muscular activity affects the pyruvic acid level.

2. Venous blood is obtained with a minimum of stasis. The tourniquet is removed immediately after the needle enters the vein.
3. Patient should not clench and open the hand before or during the drawing of the blood.
4. Withdraw 2 cc. of blood in a *cold* 2 cc. tuberculin precision syringe.
5. Eject the blood in a fine stream through the needle into 10 cc. of cold 10% trichloroacetic acid in a 15 cc. centrifuge tube.
6. Stopper with a cork, shake, and then centrifuge.
7. Store in the refrigerator until ready for analysis. The filtrates can be stored for 2 days.
8. Prepare a blank of 2 cc. of water and 10 cc. of 10% trichloroacetic acid; this blank is carried through the method simultaneously with the blood filtrate.
9. Pipette 3 cc. of the cold, clear, supernatant fluid from the centrifuged trichloroacetic acid precipitate into a small test tube (18 by 150 mm.) and place in a 25°C. water bath for 10 minutes.
10. Add 1 cc. of phenylhydrazine reagent, mix, and allow to react exactly 5 minutes.
11. Add exactly 8 cc. of ethyl acetate and pass a rapid stream of nitrogen (or air) through the mixture for 2 minutes by means of a capillary pipette.
12. Remove most of the lower aqueous layer with the capillary pipette, using a rubber bulb or suction.
13. Give the test tube a sudden circular motion to dislodge the solution adhering to the walls and remove the remaining aqueous layer.
14. Add exactly 6 cc. of 10% sodium carbonate solution to the ethyl acetate and again pass nitrogen (or air) through the mixture for 2 minutes.
15. Remove the capillary pipette and allow the ethyl acetate and aqueous solution to separate.
16. A 5 cc. pipette is inserted quickly through the upper layer (ethyl acetate); air is momentarily blown through the pipette to discharge the aqueous extract and the small quantity of ethyl acetate.
17. Draw up the aqueous solution into the pipette; remove the pipette and wipe off the tip before adjusting the contents of the pipette to the mark.
18. Place contents into a colorimeter tube, add exactly 5 cc. of 1.5 N NaOH, and r immediately.
19. Set the galvanometer at 100 with the bla using filter No. 520 and read the unknow 5 to 10 minutes after adding the alk
20. Obtain the mg. per cent of pyruvic ac from the table of values.

D. *Calibration of Standard Curve.*

1. Prepare 2 dilute standard solutions from t stock standard pyruvic acid solution (s solutions below) each time a new calibratio curve is made.
   a. For *standard A*, pipette 10 cc. of th stock standard solution into a 200 c volumetric flask and dilute to volum with water (1 cc. = 0.625 mg.).
   b. For *standard B*, pipette 10 cc. of standar A into a 100 cc. volumetric flask an dilute to volume with water (1 cc. = 0.0625 mg.).
2. Pipette the following amounts of standar solutions A and B into 100 cc. volumetri flasks and dilute to volume with water.

cc. of dilute standard solutions	Equivalent to mg. of pyruvic acid in 100 cc. of blood
2 (B)	0.125
4 (B)	0.25
8 (B)	0.5
2 (A)	1.25
4 (A)	2.5
6 (A)	3.75
8 (A)	5.0
10 (A)	6.25

3. Place 5 cc. of each of the above dilution in large test tubes, add 25 cc. of 10% tri chloroacetic acid to each tube, stopper, an mix by shaking.
4. Prepare a blank by mixing 2 cc. of wate and 10 cc. of 10% trichloroacetic acid in large test tube.
5. Pipette 3 cc. of each mixture into smal test tubes and continue as described in th method.
6. Make a sufficient number of determinations so that an average of the galvanometer readings for each tube will make a straight line when plotted on semilogarithmic graph paper.
7. Make a table of values for mg. per cent of pyruvic acid for each division on the galvanometer.

E. *Solutions.*

1. *Trichloroacetic Acid*—10%.
   a. Must be prepared frequently and kept in the refrigerator.
   b. Old solutions give increased and variable readings with the blanks.

Phenylhydrazine Reagent.

a. Grind 100 mg. of 2, 4-dinitrophenylhydrazine (Eastman No. 1866) in a mortar with increasing small volumes of approximate 2 N HCl until 100 cc. has been added to the mortar.

b. Filter through a small filter paper and keep in the refrigerator.

Sodium Carbonate Solution—10%.

a. Keep in pyrex bottles.

b. Filter, if not clear, immediately after preparation.

Sodium Hydroxide—1.5 N (keep in pyrex bottles).

Purified Ethyl Acetate.

a. Ethyl acetate ($CH_3COOC_2H_5$) may be listed under the name ethyl ethanoate.

b. Extract c.p. anhydrous ethyl acetate 3 times with 0.1 volume of a saturated solution of calcium chloride (approximately 75% at 30°C.).

c. Add 200 gm. of anhydrous sodium sulfate for each liter of ethyl acetate and let stand for several hours with frequent shaking.

d. Distill and save the fraction distilling over at 73-78°C.

e. A standardization curve must be made after each purification of the ethyl acetate.

f. The ethyl acetate used in the test may be saved and repurified.

Pyruvic Acid Stock Standard Solution.

a. Distill purified pyruvic acid (Eastman, labeled 64-47°C. at 13 mm. of Hg.) in vacuo using a water pump.

b. Collect the middle third of the distillate.

c. Weigh accurately a 50 cc. beaker and place exactly 10 cc. of the distilled pyruvic acid in it and weigh again.

d. The difference in weight will be the strength of the standard, i.e., if the 10 cc. weighs 12.5 gm., the standard will be labeled 12.5 gm. in 1 liter (1 cc. = 12.5 mg.).

e. Transfer the pyruvic acid quantitatively to a liter volumetric flask with the aid of water and dilute to volume with water.

f. This solution keeps indefinitely.

Interpretation of Blood Pyruvic Acid Findings.

Normal Values: 0.6—1.2 mg. per cent for patients in bed.

An increase in pyruvic acid means there is a decrease of vitamin B_1 or thiamine. Thiamine is activated in the liver and other organs to diphospho-thiamine which acts as a cocarboxylase and is essential in the metabolism of carbohydrates at the pyruvic acid stage. A decrease of thiamine causes an increase in pyruvic acid in the blood due to a decrease in its metabolism.

3. *Increased in:*
   Thiamine deficiency
   Fever
   Carcinoma
   Cirrhosis of the liver
   Von Gierke's disease
   Hyperthyroidism
   Cardiac decompensation
   Shock

Chemotherapy

I. Blood Sulfonamides (Bratton and Marshall's Method).

A. ***Principle:*** Para-aminobenzenesulfonamide is diazotized with nitrous acid and the excess nitrous acid is destroyed with ammonium sulfamate. The resulting diazo compound is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride producing a purplish red azo dye which can be estimated by colorimeter comparison. This reaction depends on the presence of an amino group substituted in the benzene ring and can be used for any derivative of the sulfonamide group which has the amino acid free or which can be freed by hydrolysis.

B. ***General Considerations.***

1. All the sulfonamide drugs are determined in serum.
2. The blood should be drawn just before another dose of the drug is given.
3. While sulfonamides are being used for treatment, careful check must be made on the urine for crystals to prevent overdosage and kidney damage.
4. It is desirable to have standards corresponding to the drug being determined. If in emergency it is necessary to use the standards or photoelectric curve of another drug, the results must be corrected with a factor. This factor is obtained by dividing the molecular weight of the drug being determined by the molecular weight of the drug used for a standard. For molecular weights and therapeutic blood levels, see Table 37 on page 188.
5. *Sources of error.*
   a. Not thoroughly mixing the solution after the addition of each reagent.
   b. Dye solution too old.

C. *Colorimetric Method.*

1. *Filtrate.*
   a. Pipette 12 cc. of 15% trichloroacetic acid into a 50 cc. volumetric flask.
   b. Add 2 cc. of serum shaking continuously.
   c. Dilute to mark with water, mix, let stand 20 minutes, and then filter through Whatman No. 40 filter paper.
   d. The filtrate is a 1-25 dilution.
2. *Test Proper.*
   a. Pipette 10 cc. of filtrate into a large test tube.
   b. Pipette 10 cc. of the weak standard solution (10 cc. = 0.02 mg.) into one test tube and 10 cc. of the strong standard solution (10 cc. = 0.05 mg.) into another. Use the standard corresponding to the sulfonamide being determined.
   c. Add 1 cc. of 0.1% sodium nitrite solution to each of the test tubes (standards and filtrate).
   d. Mix and let stand 5 minutes.
   e. Add 1 cc. of 0.5% ammonium sulfamate solution to each test tube.
   f. Mix and let stand 2 minutes.
   g. Add 1 cc. of dye solution to each test tube and mix.
   h. Within 30 minutes compare in a colorimeter the unknown and the standard which most nearly matches it.
   i. Set the standard at 20 mm.
3. *Calculations.*
   a. For 0.02 mg. standard use formula:

$$\frac{20}{RU} \times 0.02 \times \frac{100}{0.4} = \frac{100}{RU} = \text{mg. \%.}$$

   b. For 0.05 mg. standard use formula:

$$\frac{20}{RU} \times 0.05 \times \frac{100}{0.4} = \frac{250}{RU} = \text{mg. \%.}$$

D. *Photoelectric Colorimeter Method.*

1. *Filtrate.*
   a. Pipette 6 cc. of water into a 50 cc. volumetric flask.
   b. Add 6 cc. of 15% trichloroacetic acid.
   c. Add dropwise 1 cc. of serum and mix.
   d. Let stand 20 minutes.
   e. Add a small drop of caprylic alcohol and dilute to volume with water.
   f. Filter through Whatman No. 40 filter paper.
   g. The filtrate is a 1-50 dilution.
2. *Test Proper.*
   a. Place 10 cc. of the protein-free filtrate in a colorimeter tube.
   b. Prepare a blank by placing 8 cc. of water and 2 cc. of 15% trichloroacetic acid in a colorimeter tube.
   c. To each tube add 1 cc. of 0.1% sodium nitrite solution, mix, and let stand 5 m utes.
   d. Add 1 cc. of 0.5% ammonium sulfam solution to each, mix, and let stand 2 m utes.
   e. Add 1 cc. of dye solution to each tube a mix by whirling each tube at a 60° ang
   f. Set the galvanometer at 100 using t blank and filter No. 520.
   g. Read the unknowns within 30 minutes.
   h. Obtain the mg. per cent of sulfonami from the table of values.
3. *Calibration of Standard Curve.*
   a. Make a dilute standard solution (1 cc. = 0.01 mg.) by placing 10 cc. of the stoc standard solution (1 cc. = 0.1 mg.) in 100 cc. volumetric flask and diluting t volume with water.
   b. Make further dilutions of the dilute stand ard solution with water which has 12 cc of a 15% solution of trichloroacetic aci to each 100 cc. as follows:

cc. of dilute standard solution	Make up to	mg. in 10 cc.	mg. in 100 cc of bloo
2	100 cc.	0.002	1
4	100 cc.	0.004	2
6	100 cc.	0.006	3
8	100 cc.	0.008	4
10	100 cc.	0.010	5
12	100 cc.	0.012	6
7	50 cc.	0.014	7
8	50 cc.	0.016	8
18	100 cc.	0.018	9
10	50 cc.	0.020	10

   c. Using 10 cc. of the different dilutions instead of protein-free filtrate, proceed as described in the test proper.
   d. Perform a sufficient number of determinations on each concentration of sulfonamide so that an average of the readings will give a straight line on semilogarithmic graph paper.
   e. Make a table of values for mg. per cent for each galvanometer division.

E. *Solutions.*

1. *Standard Solutions.*
   a. *Stock standard solution.*
      1) Weigh out exactly 100 mg. of the sulfonamide desired and transfer to a liter flask. The solubility of some of the sulfonamides is such that weaker stock standard solutions must be used.
      2) Dissolve in about 800 cc. of water and then make up to volume.
      3) This stock standard solution (1 cc. = 0.1 mg.) may be kept indefinitely in the refrigerator.

b. *Working standard solutions*—make fresh every two weeks.
  1) *Weak standard solution* (10 cc. = 0.02 mg.).
    a) Transfer 2 cc. of stock standard solution to a 100 cc. volumetric flask.
    b) Add 18 cc. of 15% trichloroacetic acid and make up to volume with water.
  2) *Strong standard solution* (10 cc. = 0.05 mg.).
    a) Transfer 10 cc. of stock standard solution to a 200 cc. volumetric flask.
    b) Add 36 cc. of 15% trichloroacetic acid and make up to volume with water.

2. *Ammonium Sulfamate Solution*—0.5%.
3. *Sodium Nitrite Solution* (0.1%)—make fresh every 2 weeks from a stock 1% solution.
4. *Dye Solution.*
   a. Place 100 mg. of N-(1-naphthyl)-ethylenediamine dihydrochloride in a 100 cc. volumetric flask.
   b. Dissolve with water and dilute to volume.
   c. Keeps about 2 months in a brown bottle; do not use after it turns brown.
5. *Trichloroacetic Acid*—15%.

II. Spinal Fluid Sulfonamides.

A. General Considerations.

1. If the sulfonamide was given orally, the level will be quite low and the best dilution is a 1-10 for both visual and photoelectric colorimeter methods.
2. If the sulfonamide was given intrathecally, make the same respective dilutions of spinal fluid as for blood in both the visual and photoelectric colorimeter methods.

B. Method.

1. To 2 cc. of spinal fluid, add 4 cc. of 15% trichloroacetic acid and 14 cc. of water (1-10 dilution).
2. Let stand 20 minutes and filter.
3. Proceed as described under the method (colorimetric or photoelectric colorimeter) for blood and make the necessary correction in the final result for the new dilution.

III. Blood Bromide (Wuth's Method).

A. *Principle:* Acid gold chloride is added to a trichloroacetic acid protein-free filtrate of serum. The brown color that develops is due to the bromides, probably by replacement of the chlorine ion in the gold chloride by the bromine ion.

B. General Considerations.

1. If serum chlorides are determined when bromides are being taken, the result is total halides. Calculate per cent of chloride replacement with bromide by multiplying bromide concentration by 0.568 (the factor necessary to convert bromide levels to equivalent chloride readings) and dividing the figure so obtained by total halide concentration considered as NaCl. To calculate the serum chlorides multiply the total halides by the per cent of chloride replacement.
2. The bromide concentration of urine can be determined by the same method as for blood.
   a. Concentration in urine parallels that of blood.
   b. The excretion is quite constant and rapid after bromide therapy.
3. Bromides are used particularly in the treatment of epilepsy.

C. Method Using Permanent Standards.

1. Add 2 cc. of serum to 5.2 cc. of 10% trichloroacetic acid in a test tube.
2. Stopper and shake well.
3. Allow to stand 30 minutes and then centrifuge for 10 minutes or filter through Whatman No. 40 filter paper.
4. Place 2 cc. of the supernatant fluid or filtrate in a test tube the same size as the tubes containing the standards.
5. Add 0.4 cc. of acid brown gold chloride solution and shake.
6. Compare with the standards in a comparator box. The standards are labeled corresponding to mg. of bromide per 100 cc. of blood.
7. If reading is more than 300 mg., add 2 cc. of trichloroacetic acid and 0.4 cc. of acid brown gold chloride solution and shake. Compare with standards and multiply reading by two.
8. If the reading is below 75 mg., prepare an "O" standard by adding 0.4 cc. of acid brown chloride solution to 2 cc. of trichloroacetic acid and compare with the unknown to see if the color present is due only to the addition of the acid gold chloride solution.

D. Photoelectric Colorimeter Method.

1. Place 18 cc. of 10% trichloroacetic acid in a large test tube and add 2 cc. of serum dropwise while shaking.
2. Let stand 20 minutes and filter through Whatman No. 40 filter paper.
3. Pipette 10 cc. of the filtrate into a colorimeter tube.
4. Prepare a blank by placing 10 cc. of 10% trichloroacetic acid in a colorimeter tube.
5. Add 1 cc. of 0.5% acid brown gold chloride solution to each tube and mix.

6. Set the galvanometer at 100 using the blank and filter No. 520.
7. Read the unknown and obtain the mg. per cent of sodium bromide from the table of values.
8. *Calibration of Standard Curve.*
   a. Pipette the following amounts of the dilute standard sodium bromide solution (1 cc. = 0.5 mg.) and trichloroacetic acid-NaCl mixture into 9 colorimeter tubes.

cc. of dilute standard solution	cc. of 10% trichloroacetic acid-NaCl mixture	mg. % of sodium bromide
1	9	50
2	8	100
3	7	150
4	6	200
5	5	250
6	4	300
7	3	350
8	2	400
0	10	Blank

   b. Add 1 cc. of 0.5% acid brown gold chloride solution to each tube and read as described in the method above.
   c. Repeat a sufficient number of times so that the average of the galvanometer reading will make a straight line when plotted on semilogarithmic graph paper.
   d. Make a table of values for the mg. per cent of sodium bromide for each division on the galvanometer.

E. *Solutions.*

1. *Trichloroacetic acid*—10%.
2. *Acid Brown Gold Chloride Solution*—0.5%.
   a. The acid brown gold chloride comes in 1 gram ampules.
   b. It is not necessary to weigh the chloride but place the contents in a 200 cc. volumetric flask and dilute to volume with water, rinsing the ampule with some of the water.
3. *Permanent bromide standards* and comparator box may be obtained from La Motte, Baltimore, Maryland.
4. *Trichloroacetic Acid (10%)—NaCl (0.06%) Mixture.*
   a. Place 0.6 gm. of sodium chloride in a liter volumetric flask and add about 500 cc. of water.
   b. Add 100 gm. of trichloroacetic acid and dilute to volume with water.
5. *Standard for Photoelectric Colorimeter Method.*
   a. *Stock standard solution*—(1 cc. = 10 mg.).
      1) Weigh exactly 1 gm. of c.p. sodium bromide and place in a 100 cc. volumetric flask.
      2) Make up to volume with water.
   b. *Dilute standard solution*—(1 cc. = 0 mg.).
      1) Pipette 10 cc. of the stock standa solution into a 200 cc. volumetric fla
      2) Dilute to volume with the trichlor acetic acid-NaCl mixture.

F. *Interpretation of Blood Bromide Finding*

1. *Normal Values:* 0.8—1.5 mg. per cent.
2. The therapeutic concentration is considere about 200 mg. per cent.
3. Toxic values are usually above 250 mg. pe cent.

IV. Spinal Fluid Bromide.

A. The ratio of blood bromide to spinal flui bromide is about 3 to 1.

B. *Method Using Permanent Standards.*

1. To 4 cc. of spinal fluid in a test tube, add 0.8 cc. of 20% trichloroacetic acid.
2. Stopper and shake well.
3. Allow to stand 30 minutes and filter through Whatman No. 40 filter paper.
4. Using 2 cc. of the filtrate, carry through the same method as for blood.
5. Divide reading of standard by 3 to correct for the different dilution.

C. *Photoelectric Colorimeter Method.*

1. Make a 1-5 filtrate by adding 3 cc. of spinal fluid to 12 cc. of 10% trichloroacetic acid.
2. Proceed as described under the photoelectric colorimeter method for blood.
3. Divide the value obtained by 2 to correct for the different dilution.

V. Blood Thiocyanate (Barker's Method).

A. *Principle:* A protein-free filtrate is made from either serum or plasma by precipitation of the protein with trichloroacetic acid. The filtrate is then treated with ferric nitrate producing a red color (ferric thiocyanate) which is compared with the color produced by a standard.

B. *General Considerations.*

1. If the test for thiocyanate can not be made soon after the blood is obtained from the patient, oxalated blood can be kept in the refrigerator preserved with thymol.
2. The trichloroacetic acid filtrate is stable.
3. Thiocyanate therapy is used in hypertension.
4. Salicylates given in large amounts will give a false high thiocyanate value because they will give the same color reaction in the test as thiocyanates.

C. *Colorimetric Method.*

1. Place 5 cc. of either serum or plasma in

a centrifuge tube and add 5 cc. of 20% trichloroacetic acid.

2. Stopper and shake well.
3. Allow to stand 15 minutes, remove stopper, then centrifuge for 10 minutes or filter through Whatman No. 40 filter paper.
4. Pipette 5 cc. of the supernatant fluid or filtrate into a test tube, add 1 cc. of ferric nitrate reagent, and mix well.
5. *Standard.*
   a. To 5 cc. of each standard solution (0.5 mg., 0.35 mg. and 0.2 mg.), add 5 cc. of 20% trichloroacetic acid.
   b. Add 2 cc. of ferric nitrate reagent and mix well.
6. Compare unknown in the colorimeter with the standard that most closely approximates its color.
7. Set standard at 20 mm.
8. *Calculation:*

$$\frac{RS}{RU} \times \text{conc. of st.} \times \frac{100}{2.5} \times \frac{6}{12} = \text{mg. \%.}$$

   a. For 0.5 mg. standard use formula:

$$\frac{200}{RU} = \text{mg.\%.}$$

   b. For 0.35 mg. standard use formula:

$$\frac{140}{RU} = \text{mg.\%.}$$

   c. For 0.2 mg. standard use formula:

$$\frac{80}{RU} = \text{mg.\%.}$$

D. *Photoelectric Colorimeter Method.*

1. Prepare a serum or plasma filtrate as described under colorimetric method.
2. Prepare a blank by placing 10 cc. of 20% trichloroacetic acid in a colorimeter tube.
3. In another colorimeter tube place 1 cc. of filtrate and 9 cc. of 20% trichloroacetic acid.
4. To each tube add 2 cc. of ferric nitrate reagent.
5. Mix by shaking and read immediately after setting the galvanometer at 100 with the blank, using filter No. 520.
6. Obtain mg. per cent of thiocyanates from the table of values.
7. *Calibration of Standard Curve.*
   a. Prepare a stock standard solution (1 cc. = 1 mg. SCN) and the first dilute standard (5 cc. = 0.5 mg. SCN) of potassium thiocyanate as described under solutions.
   b. Make 2 dilutions, A and B, from the dilute standard solution.
      1) Solution A—pipette 10 cc. of the dilute standard solution into a 100 cc. volumetric flask and dilute to volume with 20% trichloroacetic acid (1 cc. = 0.01 mg.).
      2) Solution B—pipette 30 cc. of the dilute standard solution into a 100 cc. volumetric flask and dilute to volume with 20% trichloroacetic acid (1 cc. = 0.03 mg.).
   c. Set up a series of colorimeter tubes containing the following:

Tube	cc. of solutions A or B	cc. of 20% trichloroacetic acid	mg. in 100 cc. of blood
1	0.5 (A)	9.5	1
2	1.0 (A)	9.0	2
3	2.0 (A)	8.0	4.
4	3.0 (A)	7.0	6
5	5.0 (A)	5.0	10
6	8.0 (A)	2.0	16
7	10.0 (A)	0.0	20
8	4.0 (B)	6.0	24
9	5.0 (B)	5.0	30
10	0.0	10.0	Blank

   d. Add 2 cc. of ferric nitrate reagent to each tube, mix by shaking, and read immediately after setting the galvanometer at 100 with the blank, using filter No. 520.
   e. Perform a sufficient number of determinations (repeat with new stock solutions) so that an average of the readings will make a straight line when plotted on semilogarithmic graph paper.
   f. Make a table of values for mg. per cent of thiocyanate for each galvanometer division.

E. *Solutions.*

1. *Ferric Nitrate Reagent.*
   a. Dissolve 50 gm. of crystallized ferric nitrate [$Fe(NO_3)_3 \cdot 9H_2O$] in 500 cc. of water in a liter volumetric flask.
   b. Add 25 cc. of conc. nitric acid and make up to volume with water.
2. *Standard Thiocyanate Solutions.*
   a. *Stock standard solution* (1 cc. = 1 mg. SCN).
      1) Dissolve approximately 1.7 gm. of potassium thiocyanate (SCN) in 800 cc. of water.
      2) Titrate 20 cc. portions of standard silver nitrate solution (exactly 2.9195 gm. per liter) acidified with 5 cc. conc. nitric acid with the above potassium thiocyanate solution. Add 0.5 gm. of ferric aluminum sulfate (powdered ferric alum) for an indicator.
      3) Add water to the thiocyanate solution in such quantities that 20 cc. is equivalent to 20 cc. of standard silver nitrate solution.
      4) Retitrate for check.
   b. *Dilute standard solutions.*
      1) Dilute 100 cc. of the stock standard

solution to 1 liter (5 cc. = 0.5 mg. SCN).

2) Dilute 70 cc. of the stock standard solution to 1 liter (5 cc. = 0.35 mg. SCN).
3) Dilute 40 cc. of the stock standard solution to 1 liter (5 cc. = 0.2 mg. SCN).
4) These dilute standard solutions will keep 6 months.

F. *Interpretation of Blood Thiocyanate Findings.*

1. Normally there is no thiocyanate in the blood.
2. The therapeutic concentration varies from 8 to 12 mg. according to individual response with an average of 10 mg. per cent.
3. Levels should not exceed 15 mg. per cent because of the possibility of severe toxic manifestations (dizziness, headache, weakness, and nausea) which may be fatal.

VI. Blood Salicylic Acid (Brodie, Udenfriend, and Coburn's Method).

A. *Principle:* The salicylic acid is extracted from serum or plasma with ethylene dichloride in an acid medium. It is then returned to an aqueous phase as a colored iron complex which is determined in a photoelectric colorimeter.

B. *General Considerations.*

1. Serum or plasma may be kept in the refrigerator several days without appreciable loss of salicylic acid.
2. The method described below cannot be used for determination of salicylic acid in urine due to the presence of large quantities of salicyluric acid in urine.
3. Salicylates are used as a therapeutic agent in rheumatic fever.

C. *Photoelectric Colorimeter Method.*

1. Place 0.5 cc. of 6 N HCl and 30 cc. of ethylene dichloride in a 60 cc. glass-stoppered pyrex bottle.
2. Add 2 cc. of serum or plasma and shake vigorously for 5 minutes. (If the concentration is expected to be high, use 1 cc. of serum or plasma and 1 cc. of water.)
3. Transfer the mixture to a 40 cc. thick-walled centrifuge tube and centrifuge for 5 minutes at moderate speed.
4. Remove the supernatant aqueous layer by aspiration and discard.
5. Transfer exactly 20 cc. of the ethylene dichloride layer to a 60 cc. glass-stoppered bottle, add 10 cc. of water and 0.25 cc. of the iron reagent, and shake for 5 minutes.
6. Transfer at least 10 cc. of the supernata aqueous layer to a colorimeter tube.
7. Prepare a blank by placing 10 cc. of wa in a colorimeter tube and adding 0.25 c of the iron reagent.
8. Read the unknown after setting the ga vanometer at 100 using the blank and filte No. 540.
9. *Calibration of Standard Curve.*
   a. Make the following dilutions of the stoc standard salicylic acid solution with wa ter.

cc. of stock standard solution	Dilute to	Equivalent to mg. of salicylic acid
5	200 cc.	2.5
5	100 cc.	5.0
15	200 cc.	7.5
5	50 cc.	10.0
15	100 cc.	15.0
10	50 cc.	20.0
25	100 cc.	25.0

   b. Pipette 2 cc. of each standard solution into 60 cc. glass-stoppered bottles.
   c. Add 0.5 cc. of 6 N HCl and 30 cc. of ethylene dichloride to each and proceed as described in the test.
   d. Repeat tests several times on the same dilute standard solutions, then repeat on new dilute standard solutions.
   e. Average the galvanometer readings for each concentration and plot on semilogarithmic graph paper. It should make a straight line.
   f. Make a table of values according to mg. per cent of salicylic acid for each galvanometer division.

D. *Solutions.*

1. *Hydrochloric Acid*—6 N.
   a. Place about 40 cc. of water in a 100 cc. volumetric flask and add 50 cc. of conc. HCl.
   b. Cool and dilute to volume.
2. *Iron Reagent.*
   a. Place 1 gm. of ferric nitrate [$Fe(NO_3)_3$] in a 100 cc. volumetric flask.
   b. Dilute to volume with 0.07 N nitric acid.
3. *Stock Standard Salicylic Acid Solution* (1 cc. = 1 mg.).
   a. Place exactly 116 mg. of c.p. sodium salicylate in a 100 cc. volumetric flask and dilute to volume with water.
   b. This solution is stable when stored in the refrigerator.

E. *Interpretation of Blood Salicylic Acid Findings.*

1. Normally there is no salicylic acid in the blood.
2. The therapeutic blood level is considered to be at least 35 mg. per cent.
3. Salicylic acid at high blood levels will produce a hypoprothrombinemia and a moderate acidosis.

Toxicological Tests

I. Ethyl Alcohol (Levine and Bodansky's Method).

A. *Principle:* A sample of either blood or urine is absorbed on a roll of filter paper and oxidized over a potassium dichromate-sulfuric acid mixture in a tightly stoppered flask. The excess dichromate is titrated with a ferrous sulfate-methyl orange reducing solution.

B. *General Considerations.*

1. In obtaining blood from a patient, alcohol should not be used for sterilization of the skin.
2. Blood or urine can be kept for 5 days in the refrigerator if tightly stoppered without appreciable loss of alcohol.

C. *Method.*

1. Pipette 10 cc. of standard potassium dichromate solution and 10 cc. of conc. sulfuric acid into the bottom of a 125 cc. Erlenmeyer flask. Care must be exercised so that none of the reagents drip onto the neck or sides of the flask.

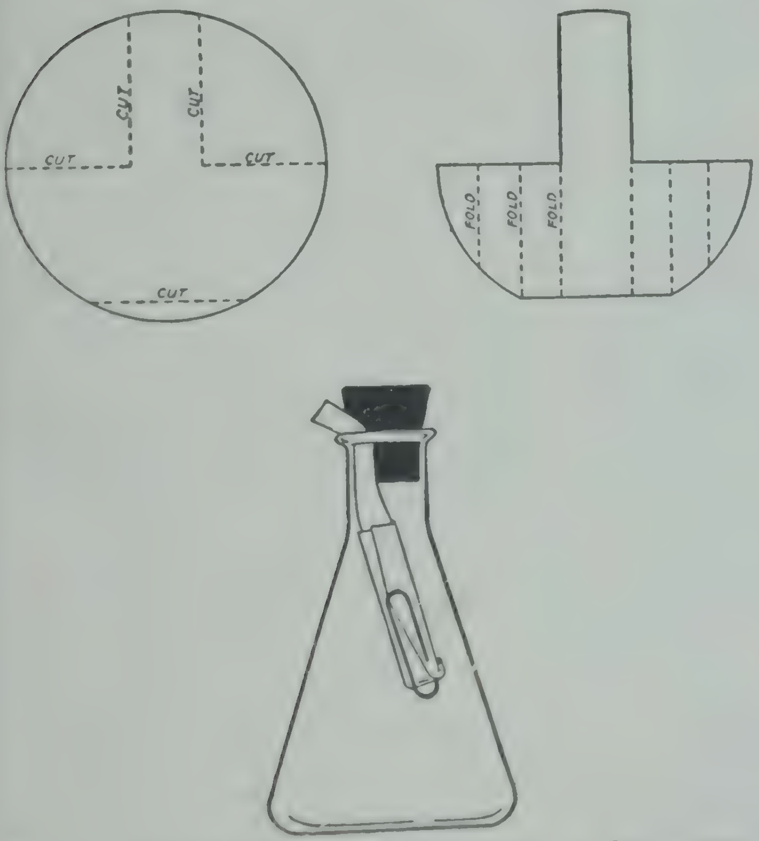


Fig. 32. Method of preparing the roll of filter paper for the determination of ethyl alcohol. (Levine, H. and Bodansky, M. A., Technical Supplement of the Am. Jour. of Clin. Path., published by Williams and Wilkins, Baltimore.)

2. This mixture of reagents will determine alcohol concentrations up to 200 mg. per 100 cc. in either blood or urine when 0.5 cc. samples are used and concentrations up to 500 mg. per 100 cc. with 0.2 cc. samples.
3. Prepare a roll of Whatman No. 2 filter paper (11 cm.) in the manner indicated diagrammatically in Fig. 32.
4. Into the small cup formed by the rolled paper, pipette either 0.5 cc. or 0.2 cc. portions of blood or urine.
5. Suspend the roll of filter paper over the potassium dichromate oxidizing reagent by inserting the strip of paper attached to the roll between the neck of the flask and the rubber stopper (Fig. 32). Care should be taken to prevent the filter paper from touching the acid mixture.
6. Press the stopper tightly into the neck of the flask.
7. In a similar manner prepare a blank using the same volume of water in place of blood or urine.
8. Place the flasks in a drying oven kept at approximately 100°C. for 30 minutes. If an oven is not available, the flasks may be placed in boiling water for 30 minutes.
9. Allow the flasks to cool to room temperature.
10. Carefully remove the stopper and roll of filter paper and titrate the contents of the flasks with the red reducing fluid using a 5 cc. microburette graduated in 0.01 cc.
11. The first permanent pink color obtained is the end-point.
12. After titrating one of the flasks to the end-point, add 5 cc. of the standard potassium dichromate solution and again titrate.
13. The amount of red reducing fluid required will express the equivalence of the reducing fluid to the dichromate solution.
14. The quantity of reducing fluid required to titrate the 5 cc. of the dichromate solution should be about 2.5 cc.
15. *Calculation:*
    Since 5 cc. of the standard dichromate solution is completely reduced by 0.5 mg. of alcohol, twice the amount of the red reducing fluid required to titrate 5 cc. of the dichromate is equivalent to 1 mg. of alcohol.

$$\frac{B-A}{2C} \times \frac{100}{S} = \text{mg.}\%.$$

A = cc. of reducing fluid required to titrate the unknown.
B = cc. of reducing fluid required to titrate the blank.
C = cc. of reducing fluid required to titrate the added 5 cc. of dichromate solution.
S = cc. of sample taken for analysis.

D. *Solutions.*

1. *Standard Potassium Dichromate Solution* (1 cc. = 0.1 mg. alcohol).
   a. Place 0.4258 gm. of c.p. potassium dichromate ($K_2Cr_2O_7$) in a liter volumetric flask.
   b. Dissolve in water and make up to volume.
2. *Methyl Orange*—0.1%.
   a. Place 1 gm. of methyl orange in a liter volumetric flask.
   b. Make up to volume with approximately 0.025 N NaOH and filter.
3. *Ferrous Sulfate Solution*—20%.
   a. Dissolve 50 gm. of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 150 cc. of water in a 250 cc. volumetric flask.
   b. Add 30 cc. of conc. sulfuric acid and dilute to volume with water.
   c. If kept in a well stoppered bottle, the solution will not deteriorate for 1 year.
4. *Red Reducing Fluid.*
   a. To 35 cc. of 1:1 sulfuric acid, add 15 cc. of 0.1% methyl orange solution and 1 cc. of 20% ferrous sulfate solution.
   b. Mix and allow to cool to room temperature before using.
   c. This solution keeps for 3 to 4 days.

E. *Interpretation of Blood and Urine Alcohol Findings.*

1. The interpretation of blood and urine alcohol findings depends entirely upon the time the specimen was obtained in relation to the time the alcohol was imbibed.
2. Values up to 50 mg. per cent in blood are not conclusive evidence the individual has been drinking, since there are small amounts of reducing substances normally present in blood.
3. Values of 100 to 150 mg. per cent in blood are evidence that the individual is under the influence of alcohol but not definitely intoxicated.
4. The level of 150 mg. per cent in blood is considered indicative of intoxication; however, there is a wide range of tolerance so that in some individuals intoxication may be reached at lower or higher levels.
5. A level of 200 mg. per cent in blood is considered a definite indication of intoxication.
6. When values of 500 mg. per cent are reached, the person is probably in coma and death may result.
7. These values cannot be applied to urine b cause the urine alcohol may be higher o lower than the blood alcohol depending up the stage of absorption or elimination o alcohol.

II. Barbiturates in Urine.

A. *Principle:* The barbiturates are extracte with chloroform and the reddish-purpl color produced on the addition of cobal acetate and isopropylamine is compared wit the color similarly produced in a standar solution of barbital.

B. *General Considerations.*

1. The sulfonamide compounds interfere with the color reaction in the test for barbiturates.
2. All solutions for this test must be kept water-free.
3. If very small amounts of barbiturates are expected, a larger quantity of urine can be evaporated to dryness over a steam or sand bath.

C. *Method.*

1. Place 25 or 50 cc. of urine in a 400 cc. beaker and make acid with 10% sulfuric acid.
2. Evaporate just to dryness over a steam or sand bath.
3. Add 20 cc. of chloroform to the residue and evaporate to about 10 cc.; then filter through Whatman No. 50 filter paper into a 100 cc. beaker.
4. Repeat this extraction of the residue twice.
5. Evaporate the combined extracts to about 5 cc., add about 0.25 gm. of activated charcoal, and mix to remove the pigment.
6. Filter through Whatman No. 50 filter paper into a 100 cc. beaker, rinsing the first beaker with five 2 cc. portions of chloroform.
7. Evaporate the combined washings to about 2 cc. and transfer quantitatively to a graduated centrifuge tube with the aid of small portions (0.5 cc.) of chloroform.
8. Dilute to 5 cc. with chloroform.
9. Prepare 2 standards as follows:
   a. In tube 1 place 4 cc. of standard barbital solution (2 mg.) and 1 cc. of chloroform.
   b. In tube 2 place 2 cc. of standard barbital solution (1 mg.) and add 3 cc. of chloroform.
10. Add to all tubes 0.25 cc. of 1% cobalt acetate solution and 1.5 cc. of 5% isopropylamine solution and mix.
11. Compare at once in the colorimeter with the standard which most nearly approxi-

mates the color.

12. Set the standard at 20 mm.
13. *Calculation:*

$$\frac{RS}{RU} \times 2 \text{ (or 1)} \times \frac{100}{25 \text{ (or 50)}} = \text{mg.\%}.$$

D. *Solutions.*

1. *Sulfuric Acid*—10%.
2. *Cobalt Acetate Solution*—1%.
   - a. Place 1 gm. of cobalt acetate [$Co(C_2H_3O_2)_3$] in a 100 cc. volumetric flask.
   - b. Make up to volume with absolute methyl alcohol.
3. *Isopropylamine Solution*—5%.
   - a. Pipette 5 cc. of isopropylamine (Eastman) into a 100 cc. volumetric flask and dilute to volume with absolute methyl alcohol.
   - b. Keep in a brown glass-stoppered bottle.
4. *Standard Barbital Solution* (1 cc. = 0.5 mg.).
   - a. Place exactly 50 mg. of barbital in a 100 cc. volumetric flask.
   - b. Dilute to volume with chloroform.

E. *Interpretation of Urinary Barbiturates.*

1. Normally there should be no barbiturates present.
2. Found after taking large doses of sedatives containing barbital or one of its derivatives.

III. Arsenic in Urine (Gutzeit's Qualitative Method).

A. *Principle:* In the presence of zinc, hydrochloric acid, and iodine, arsenic is converted to arsine (AsH_3) which is volatile. This reacts with the silver nitrate on the filter paper to produce a silver arsenide compound which is yellow. A drop of water placed on the yellow filter paper will produce a black color probably due to free silver from the oxidation of the arsenide to arsenate.

B. *General Considerations.*

1. Do not use pyrex glassware.
2. All solutions must be arsenic-free.

C. *Method.*

1. Place about 1 gm. of arsenic-free zinc (preferably granular) in a large test tube.
2. Add 5 to 10 cc. of pure dilute HCl (1-5 dilution) and a few drops of Gram's iodine solution.
3. Cover the mouth of the test tube with a filter paper cap moistened with concentrated aqueous solution of silver nitrate (1 gm. $AgNO_3$ in 1 cc. water).
4. If no color appears on the cap after a few minutes, the reagents are free of arsenic.
5. Add 5 to 10 cc. of urine, cover at once with the same cap, and allow to react for several minutes.
6. If arsenic is present, the paper will become lemon yellow which will turn black upon the addition of a drop of water.
7. Normally no arsenic is found in urine.

IV. Mercury in Urine (Biancalani's Qualitative Test).

A. *Principle:* Mercury is deposited on copper wire in strongly acid urine. Upon heating the wire, the mercury vapor forms a red precipitate of $HgI_2 \cdot CU_2I_2$ when it comes in contact with freshly precipitated cuprous iodide.

B. *General Considerations.*

1. A 24 hour specimen of urine should be used and this may be concentrated to a smaller amount over a water bath.
2. The copper wire should be cleaned by placing it in warm nitric acid and then thoroughly rinsing with water. Handle with forceps after it is cleaned.
3. Test is sensitive to 0.005 mg. of mercury bichloride per 100 cc. of urine.
4. Normally the test should be negative.

C. *Method.*

1. Make the urine strongly acid with dilute sulfuric acid (1-5 dilution).
2. Immerse a clean coiled copper wire in the urine and heat on a steam bath for 1 hour.
3. Remove the wire, dry, and place in a pyrex test tube.
4. Place a strip of filter paper coated with freshly precipitated cuprous iodide over the mouth of the tube.
5. Heat strongly the part of the test tube containing the wire.
6. If mercury is present, its vapor will deposit a salmon to rose color compound ($HgI_2 \cdot Cu_2I_2$) on the paper containing the white cuprous iodide.
7. A clean copper wire should be heated in a similar manner to check the reagent.

D. *Cuprous Iodide.*

1. Dissolve 5 gm. of crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$) and 3 gm. of anhydrous ferrous sulfate ($FeSO_4$) in 100 cc. of water.
2. While stirring add 50 cc. of water in which 7 gm. of potassium iodide has been dissolved.
3. Filter and wash the white precipitate (Cu_2I_2) on the filter paper with water until all the iodine is removed.
4. This cuprous iodide may be kept for a short time in the form of a suspension in a brown glass bottle by transferring it to the bottle with the aid of a small amount of water.

ALLERGY EXTRACTS

Extracting and Diluting Solutions

I. Solutions.

A. ***Buffered Dextrose-Phenol Solution.***

Dextrose, c.p.	45 gm.
Phenol, c.p.	5 gm.
Sodium bicarbonate, c.p.	2 gm.

Place in a liter volumetric flask and fill to the mark with distilled water.

B. ***Coca's Solution.***

Sodium chloride, c.p.	5.00 gm.
Sodium bicarbonate, c.p.	2.75 gm.
Phenol, c.p.	4.00 gm.

Place in a liter volumetric flask and fill to the mark with distilled water.

II. Use of Solutions.

A. When the above solutions are used for diluting sterile extracts, they are first passed through a sterile Seitz filter and cultured for sterility as described under pollen extract.

B. The same solution is used in making dilutions as was used in making the extract.

Extracts

I. Pollen Extract.

A. ***Preparation of Stock Solution (3%).***

1. Extract 3.5 gm. of pollen with 50 cc. of ether for 24 hours at room temperature.
2. Filter off the ether and return any pollen on the filter paper to the flask.
3. Extract again with ether for 24 hours and filter.
4. Dry the pollen on the filter paper until all fumes of the ether have disappeared.
5. Place 3 gm. of the extracted pollen in the same flask which has been dried and freed from the odor of ether.
6. Add 100 cc. of buffered dextrose-phenol solution or 100 cc. of Coca's solution and glycerin (33.3 cc. of Coca's solution plus 66.7 cc. of c.p. glycerin).
7. Add a few glass beads and then stopper.
8. Keep in the refrigerator and shake for a few minutes at hourly intervals for 2 or 3 days.
9. Filter through filter paper and then through a sterile Seitz filter.
10. Culture the filtrate for sterility by placing 0.5 cc. into a tube of thioglycollate glucose broth.
11. If there is no growth after 72 hours incubation, place the extract in sterile 30 cc. vaccine bottles.

B. ***Use of Extract.***

1. The 3% stock solution is used for scratch tests.
2. Dilutions for intradermal tests and for treatment are made as follows:
   - a. One part stock solution plus 2 parts of diluent = 1-100 dilution.
   - b. One part of the 1-100 dilution plus 9 parts of diluent = 1-1000 dilution.

II. House Dust Extract.

A. ***Preparation of Stock Solution.***

1. Place the house dust in a jar and add toluene until the dust is well covered.
2. Shake thoroughly and let stand for 1 hour.
3. Filter off the toluene and let dust dry on the filter paper until all fumes of toluene have disappeared (1 to 2 days).
4. Weigh the dust and for each 20 grams add 50 cc. of buffered dextrose-phenol solution and 50 cc. of water.
5. Mix well and let stand for 48 hours.
6. Squeeze through a towel and then filter through filter paper.
7. Evaporate the filtrate to one-half volume by placing in a Visking sausage casing and hanging the casing on a nail or ring stand. Evaporation can be hastened by using an electric fan.
8. Filter through filter paper and pass through a sterile Seitz filter.
9. Test for sterility by placing 1 cc. in a tube of thioglycollate glucose broth and incubating for 72 hours.
10. If no growth appears, place the solution in sterile 30 cc. vaccine bottles.

B. ***Use of Stock Solution.***

1. The stock solution is used for scratch tests.
2. Dilutions of 1-10, 1-100, 1-1000 and 1-10,000 are made for intradermal tests and for treatment.

III. General Extracts.

General Considerations.

All materials to be extracted should be in the raw or natural state.

They must be either powdered or ground in a meat grinder as fine as possible in order to increase the surface area on which the extracting fluid can act and to break down cell membranes.

Materials containing fat must be defatted with ether or toluene before making extracts. (Carbon tetrachloride may be used to remove resinous material but must in turn be removed with ether.)
  a. Add ether until there is a 2 inch layer above the material.
  b. Shake several times during the day and change ether daily until it is clear.
  c. After the last extraction, dry the material until all fumes of the ether have disappeared (1 to 3 days).

The materials are extracted with one of the extracting solutions.
  a. Buffered dextrose-phenol solution has proved better than Coca's solution.
  b. Weigh the material to be extracted, place in a wide-mouth bottle with a tight-fitting screw top, and add the extracting solution in the proportion given under each group of materials.
  c. Shake for a few minutes at hourly intervals and leave at room temperature for 48 hours or in the refrigerator for 72 hours.
  d. Filter through filter paper before passing through a Seitz filter for sterilization.

5. Extracts of some materials must be dialyzed to remove irritating elements, dyes, and some electrolytes.
  a. Place the extract, after filtering through filter paper, in a Visking sausage casing.
  b. Place the casing and its contents in a beaker containing the solution used in making the extract.
  c. Change the solution in the beaker several times. If the solution becomes discolored, it must be changed until the color is negligible.
  d. Care must be taken not to dialyze too long in order not to lose some of the potency of the extract.

6. All stock solutions must be tested for sterility after passage through a Seitz filter; place 1 cc. in a tube of thioglycollate glucose broth and incubate at 37°C. for 3 days.

7. When dilutions of 1-1000 are needed, first prepare a 1-100 dilution of the stock solution and then a 1-1000 dilution.

8. All new extracts should be tested for potency and irritating substances before using for diagnosis.
  a. The extract should give a positive skin reaction on a person known to react to the substance used.
  b. It should give a negative reaction on a person known not to react to the substance used in order to rule out the presence of irritating substances in the extract.

9. Dilutions for intradermal tests are dilutions of the original material so that the extraction dilution must be considered.

B. *Meat and Fish.*

1. Fresh meat is obtained with as little blood as possible.
2. Remove any connective tissue present and grind in a meat grinder.
3. Defat with ether or toluene and dry.
4. Extract with buffered dextrose-phenol solution in the proportion of 1 part of dried meat to 4 parts of solution, except veal, chicken, lamb, pork, and shrimps which are extracted in the proportion of 1 to 9.
5. Beef and beef liver extracts should be dialyzed.
6. A 1-1000 dilution is used for intradermal tests.
7. Make fresh extracts every six months.

C. *Cereals.*

1. Grind and extract 1 part with 9 parts of buffered dextrose-phenol solution under a thin layer of toluene.
2. Dialyze the extracts of wheat, rye, oats, and buckwheat for 24 to 48 hours.
3. A 1-100 dilution is used for intradermal tests.
4. Make fresh extracts once a year.

D. *Seeds, Condiments, Beverages, Spices, and Nuts.*

1. If not obtained in a powdered form, either chop or grind the material.
2. Defat those containing fat with ether or toluene and dry.
3. Extract 1 part with 9 parts of buffered dextrose-phenol solution, except filberts, almonds, and English walnuts which are extracted in the ratio of 1 part to 4 parts of solution.
4. Dialyze all the extracts except those of nuts and cottonseed.

5. A 1-100 dilution is made for intradermal tests, except cottonseed and flaxseed which are diluted 1-1000.
6. Make fresh extracts every 6 months.

E. ***Vegetables.***

1. Obtain vegetables when fresh in season.
2. Grind in a meat grinder and extract with buffered dextrose-phenol solution in the following proportions:
    a. One part of cabbage, cauliflower, string beans, endive, asparagus, and white potato to 4 parts of solution.
    b. One part of onion, beet, spinach, carrot, celery, and sweet potato to 2 parts of solution.
    c. One part radish, lima beans, peas, soy beans, lettuce, red and green peppers, and parsley to 9 parts of solution.
3. A 1-25 dilution of each is used for intradermal tests, except peas and soy beans which are used in 1-100 dilutions.
4. Make fresh every 6 months.

F. ***Fruits.***

1. Obtain fruits when fresh in season.
2. Grind in a meat grinder or, for those having a large amount of juice, squeeze the juice out as completely as possible.
3. Defat coconut, date, olive, and banana with ether or toluene and dry.
4. Extract with buffered dextrose-phenol solution under a thin layer of toluene in the following proportions:
    a. One part of tomato, plum, raspberry, and watermelon to 1 part of solution.
    b. One part of cranberry to 2 parts of solution.
    c. One part of apple, strawberry, grape, apricot, peach, pear, cantaloupe, cucumber, lemon, orange, grapefruit, coconut, date, banana, and pineapple to 4 parts of solution.
    d. One part of cherry, fig, and olive to 9 parts of solution.
5. For intradermal tests make the following dilutions:
    a. A 1-3 dilution of watermelon.
    b. A 1-10 dilution of tomato, apple, apricot, peach, plum, raspberry, strawberry, coconut, date, banana, and pineapple.
    c. A 1-25 dilution of cherry, pear, lemon, orange, grapefruit, fig, and olive.
6. Make fresh extracts every 6 months.

G. ***Eggs.***

1. Separate the yolks from the whites of fresh eggs.
2. Extract 1 part of each with 9 parts of bu fered dextrose-phenol solution.
3. A 1-1000 dilution of egg white and a 1-10 dilution of egg yolk are used for intraderm tests.
4. Make fresh extracts every 6 months.

H. ***Milk.***

1. Obtain 800 cc. of fresh nonpasteurized milk from which the fat has been removed.
2. Add 2.5 cc. of a 1% rennin solution and place in a water bath at 37°C. for 30 minutes without stirring.
3. Strain through a sterile towel to remove the precipitated casein.
4. Add 7 cc. of a saturated solution of sodium carbonate to the filtrate and sterilize by filtering through a Seitz filter.
5. For intradermal tests dilute 1 part of milk extract with 1 part of solution.
6. Make a fresh extract every 6 months.

I. ***Milk Products.***

1. Casein, cheese, and buttermilk can be obtained in powdered form for extraction.
2. Defat with ether or toluene and dry.
3. Extract with buffered dextrose-phenol solution in the following proportions:
    a. One part cheese with 4 parts of solution.
    b. One part casein, lactalbumin, and buttermilk to 9 parts of solution.
4. A 1-100 dilution is used for intradermal tests.
5. Make fresh extracts every 6 months.

J. ***Epidermals.***

1. Obtain hair directly from the animal by combing or currying.
2. Obtain feathers directly from the fowl by dry plucking.
3. Care must be taken to avoid water coming in contact with the epidermals and to avoid bloody or soiled material.
4. Cut the substance into small pieces and defat.
5. Extract one part with 9 parts of buffered dextrose-phenol solution under a thin layer of toluene.
6. For intradermal tests make the following dilutions:
    a. A 1-100 dilution for camel hair, chicken, duck, goose, and turkey feathers.
    b. A 1-200 dilution for rabbit hair.
    c. A 1-1000 dilution for cat, cattle, dog, goat, and hog hair, horse dander, and sheep wool.
7. Make fresh extracts once a year.

Silk.

Obtain the silkworm in the pupa stage and remove the silkworm from the cocoon.

Grind the dry worm in a mortar and defat with ether or toluene.

Extract 1 part with 9 parts of buffered dextrose-phenol solution.

Dialyze for 24 hours.

A 1-1000 dilution is used for intradermal tests.

Make a fresh extract once a year.

Miscellaneous.

Kapok extracts can be made from either the seed or fiber; if the seed is used, it must be defatted.

Henna, pyrethrum, and orris root can be obtained in powdered form.

3. Hops, sand flies, tobacco, rayon, and karaya gum extracts are made from the raw material.
4. Grind or cut into small pieces.
5. Defat all substances containing fat with ether or toluene.
6. Extract 1 part to 9 parts of buffered dextrose-phenol solution under a thin layer of toluene, except pyrethrum which is extracted 1 part to 4 parts of solution and karaya gum which is extracted 1 part to 49 parts of solution.
7. Dialyze all extracts except kapok, rayon, orris root, and karaya gum.
8. For intradermal tests make a 1-100 dilution of all except pyrethrum, sand flies, orris root, and karaya gum which are used in a 1-1000 dilution.
9. Make fresh extracts once a year.

Tissue Sectioning

I. Fixation.

A. *General Considerations.*

1. For the preparation of satisfactory sections, tissues must be "fixed" immediately after removal from the body.
2. Fixing reagents possess the properties of penetrating, killing, preserving, and hardening tissues.
3. The choice of a proper fixing reagent depends upon the nature of the tissue, the suspected pathological lesion, and the stain to be used.
4. Tissues for fixation should be cut in pieces not more than 5 mm. in thickness to permit rapid penetration and fixation.

B. *Fixing Reagents.*

1. *Zenker's Fluid.*
   a. This is the best fixative for general histologic study and for preservation of nuclear structure, bacteria, and fibrils.
   b. Fix tissues in acetic-Zenker's or formol-Zenker's fluid for 12 to 24 hours.
   c. Wash in running water 12 to 24 hours.
   d. Transfer to 35% alcohol containing enough Lugol's solution to produce a port wine color for 12 to 18 hours to remove any precipitated mercury. (This step may be omitted, but the precipitated mercury must be removed later from the cut sections before staining.)
   e. Preserve in 70% alcohol.
   f. *Stock Zenker's fluid.*

Potassium dichromate	25 gm.
Mercury bichloride	50 gm.
Distilled water to make 1 liter.	

   Dissolve the salts in the water with the aid of heat.

   g. *Acetic-Zenker's fluid.*
      1) Add 5% (by volume) of glacial acetic acid to the stock Zenker's fluid.
      2) The acetic acid must be added at the time the fluid is to be used and only to that portion of the stock solution which is required on any given occasion.
   h. *Formol-Zenker's fluid* (Helly's fluid
      1) Add 5% (by volume) of neutr formalin to the stock Zenker's
      2) The formalin must be added a time the fluid is to be used and to that portion of the stock sol which is required on any given sion.
2. *Neutral Formalin—10%.*
   a. This solution is cheaper, more easily pared, fixes and hardens tissues qui and permits a large variety of stai methods.
   b. Fix tissues in 10% formalin for 2 48 hours and then preserve in 70% hol.
   c. To prepare use 1 part of aqueous mercial formalin (40% solution of maldehyde) to 9 parts of water and tralize by adding calcium carbonate (ble chips) or lead oxide.
3. *Bouin's Fluid.*
   a. This fluid is used for general pur and for special study, particularly of bryos.
   b. Fix tissues in Bouin's fluid up to 18 h followed by washing in 50% alc
   c. The remaining yellow color can be tracted from the tissues (or embryos placing them in 70% alcohol contai 2 to 3 cc. of a saturated solution of ium carbonate to 100 cc., or subseque extracted from the sections on the by placing them in a 10% solutio lithium carbonate.
   d. *Bouin's fluid.*

Picric acid, saturated aqueous solution (about 1.22%)	
Formalin	
Acetic acid, glacial	

4. *Alcohol.*
   a. A fixative which hardens and dehyd tissues at the same time.
   b. Bacteria, fibrin, muscles, pigments, elastic fibers stain well after fixatio alcohol.

c. It is used for fixing tissues to be stained for phosphatase.
d. Place tissues first in 80% alcohol and in 2 to 4 hours change to 95% alcohol.
e. After fixation with any of the fixatives, tissues can be stored in 70% alcohol indefinitely.
f. Absolute alcohol is the only fixative to be used for the preservation of glycogen.
   1) The tissue must not come in contact with water until after embedding.
   2) It is placed directly into chloroform from absolute alcohol.

II. Decalcification.

. *Method.*

. Bone and other calcified tissues must be decalcified before sections can be prepared.

. Cut or saw sections into slices 2 to 5 mm. thick before fixation.

. Fix and harden thoroughly in 10% neutral formalin or in formol-Zenker's fluid.

. Place in one of the decalcifying reagents and change the fluid frequently. The process should not take more than 3 to 4 days.

. Remove the decalcifying reagents by washing in running water for 6 to 12 hours.

. Harden the tissues in alcohol and embed.

. *Decalcifying Reagents.*

Nitric Acid—5%.

Sulfurous Acid—a saturated aqueous solution of sulfur dioxide (6-7%).

Trichloroacetic Acid—5%.

Picric Acid—a saturated aqueous solution.

III. Embedding and Cutting Methods.

. *General Considerations.*

Fixed tissues require some medium to furnish stability and hold cellular structures in proper relation to each other before they can be sectioned.

Two substances are used for embedding—paraffin and celloidin.

Celloidin is used for specialized work, such as sectioning of eyes, and it requires at least one week to embed tissue.

. *Paraffin Method.*

. Wash the fixed tissue in water to remove the fixative.

. Cut in pieces about 2 mm. in thickness orienting it in such a way that the section will be in a plane desired for study.

. Place in 80% alcohol for 4 hours.

. Change to 95% alcohol for 4 hours.

. Leave in 100% alcohol overnight.

. Place in chloroform 2-4 hours.

7. Place in 50% solution of paraffin in chloroform 2-4 hours in closed jars in a 37°C. oven.
8. Place in paraffin in a 56°C. oven for 2-4 hours.
9. Block the tissue in the following manner:
   a. Fill the mold with melted paraffin.
   b. Transfer the tissue from the infiltration bath of melted paraffin to the mold with forceps.
   c. Drop paper labels into molds and cool by placing molds in the refrigerator or immersing in cold water.
10. When blocks have hardened, trim and place in labeled boxes. Keep blocks in the refrigerator until sectioned.
11. For routine examination cut sections with a rotary or sliding microtome at 5-7 microns in thickness.
12. Float the sections on the surface of warm distilled water (about 38°C.). Avoid bubbles and stretch to remove wrinkles.
13. Mount each section on a clean slide which has been prepared with a thin smear of Mayer's albumin-glycerin fixative, by immersing the slide under the floating section. (Mayer's albumin-glycerin fixative—mix 50 cc. glycerin and 50 cc. egg albumin, filter, add a small thymol crystal, and store in the refrigerator.)
14. Lift the slide with the section on it, drain for a few seconds, place on a warming pan adjusted to 40°-50°C., and leave at least 1 hour or overnight.
15. Stain slides.

IV. Frozen Sections.

A. *General Considerations.*

1. Frozen sections are of great value when a quick diagnosis is required while the patient is under an anesthetic and a question of malignancy must be decided in a few minutes.
2. They may be made with unfixed or fixed tissue; the embedding process is eliminated.
3. They must be used for certain special stains such as for fat.

B. *Freezing Tissue and Cutting Sections.*

1. A small block of tissue not more than 5 mm. in thickness is prepared.
2. If the tissue has not been previously fixed, heat for about 1 minute at 80°C. in a beaker partially filled with 10% formalin.
3. Rinse tissue in water and place on the stage of the freezing microtome.
4. Freeze tissue thoroughly, then allow tissue to thaw out just enough to obtain proper

consistency, and cut sections 8-10 microns in thickness.

5. Wipe sections from knife and float on distilled water.

C. *Hematoxylin and Eosin Stain.*

1. Cut frozen sections.
2. Use a bent glass rod to transfer section to filtered hematoxylin and stain for 30 seconds or longer.
3. Rinse in tap water.
4. Decolorize in acid alcohol.
5. Wash quickly in tap water.
6. Transfer to dilute ammonia water until section appears blue.
7. Rinse in tap water and float section on slide.
8. Blot with bilulous paper.
9. Using a medicine dropper, flood the slide, which is held almost horizontally, with the following solutions in order:
   Eosin Y in 90% alcohol
   80% alcohol
   95% alcohol
   100% alcohol
   Carbol-xylol
   Xylol
10. Mount in balsam, clarite, or permount.
11. For results and solutions, see hematoxylin and eosin stain under staining methods.

D. *Thionin Stain.*

1. Cut frozen sections.
2. Use a glass rod to transfer section to the thionin solution and stain for a few seconds.
3. Wash in tap water.
4. Float section on slide and examine.
5. *Thionin Solution.*

Thionin (total dye content 91%)	0.5 gm.
Alcohol (20%)	100 cc.

6. This stain may be used with unfixed tissue, but tissue fixed in formalin is preferable.
7. *Results:*
   a. Nuclei stain blue to purple.
   b. Collagen stains reddish.
   c. Elastic fibers stain light green.

E. *Polychrome Methylene Blue Stain.*

1. Cut frozen sections.
2. Use a glass rod to transfer section to the polychrome methylene blue solution and stain for 1-2 seconds.
3. Wash in tap water.
4. Float section on slide and examine.
5. *Polychrome Methylene Blue Solution*—1% aqueous solution made fresh once a month.
6. This stain may be used with unfixed tissue, but tissue fixed in formalin is preferable.
7. *Results:*
   a. Nuclei stain blue to purple.
   b. Remaining tissue stains reddish.

F. *Fat Stain* (Sudan IV).

1. Cut frozen sections.
2. Dip in 70% alcohol.
3. Stain in Sudan IV—3 to 5 min.
   (Staining should be done in a covered ve
   to prevent precipitation due to evapora
   of acetone.)
4. Wash quickly in 70% alcohol.
5. Wash in water.
6. Stain nuclei in hematoxylin (Harris' so
   tion)—1 to 2 min.
7. Wash in water.
8. Mount in glycerin (temporary mount)
   glycerin jelly.
9. *Results:*
   a. Fat stains orange to red and choleste
      less brilliantly red; normal myelin a
      fatty acids do not stain.
   b. Nuclei stain blue.
10. *Sudan IV Stain* (scarlet red).

Scarlet red	1 g
Alcohol (70%)	50
Acetone, c.p.	50

Keep in a tightly stoppered bottle and fil
before using.

V. Staining Methods.

A. *Hematoxylin and Eosin Stain.*

1. Place sections fixed on slides in a Cop
   jar or other staining dish.
2. Remove paraffin with two changes of xy
   —5 min. each.
3. Absolute alcohol—5 min.
4. 95% alcohol—3 min.
5. 80% alcohol—3 min. (If tissues have be
   fixed with Zenker's solution, add enou
   Lugol's solution to the 80% alcohol to gi
   a port wine color and leave tissues in
   for 5 minutes, after which they are plac
   in a 5% solution of sodium thiosulfate for
   few seconds.)
6. Tap water—3 min.
7. Stain in hematoxylin solution—5 to 8 mi
   (filter stain before using).
8. Wash the stained sections thoroughly
   running tap water.
9. Decolorize in acid alcohol until sections a
   of a reddish hue.
10. Wash thoroughly in tap water and plac
    sections in dilute ammonia water until th
    bluish purple color is restored.
11. Wash in tap water.
12. 90% alcohol—5 min.
13. Counterstain in eosin solution—1/2 to
    min.
14. Rinse in 95% alcohol.
15. 95% alcohol—3 min.
16. Absolute alcohol, 2 changes—5 min. eac

17. Carbol-xylol—2 min.
18. Clear in xylol, 2 changes—5 min. each.
19. Mount in Canada balsam, clarite, or permount.
20. Label slide on left side.
21. *Results:*
    a. The nuclear structures retain the basic dye of hematoxylin and remain blue.
    b. The cytoplasm stains red with the eosin.
    c. Connective tissue fibers stain red.
22. *Staining Solutions.*
    a. *Hematoxylin (Harris') solution.*

Hematoxylin	5.0 gm.
Alcohol (95%)	50.0 cc.
Alum (ammonium or potassium)	100.0 gm.
Distilled water	1000.0 cc.
Mercuric oxide	2.5 cc.

1) Dissolve the hematoxylin crystals in the alcohol in a beaker.
2) Dissolve the alum in water in a 2 liter pyrex flask with the aid of heat and add the hematoxylin solution.
3) Bring to a boil as rapidly as possible and then add the mercuric oxide.
4) The solution assumes a dark purple color.
5) Remove the vessel containing the solution from the flame and cool immediately by plunging into cold water.
6) Filter before using.

b. *Eosin solution.*

Eosin Y (alcohol-water soluble)	2.5 gm.
Alcohol (90%)	1000.0 cc.
Hydrochloric acid (0.1 N)	40.0 cc.

1) Dissolve eosin in the alcohol.
2) Add the hydrochloric acid which enhances the staining reaction.

c. *Acid alcohol*—0.25% hydrochloric acid in 70% alcohol.

d. *Dilute ammonia water*—5 drops of ammonium hydroxide to 100 cc. of distilled water.

e. *Carbol-xylol*—dissolve 1 part of melted carbolic acid crystals (phenol) in three parts of xylol.

B. *Iron Hematoxylin (Heidenhain's) Stain.*

1. Tissues fixed in any of the above fixatives stain well by this method.
2. Carry paraffin sections through xylols, alcohols, and water as described under hematoxylin and eosin stain.
3. Mordant the deparaffinized sections in ammonium ferric alum solution for 3 to 12 hours.
4. Wash in running water for 1 hour.
5. Stain for 1 to 3 hours in 1% hematoxylin solution.
6. Wash in running water for 30 minutes.
7. Differentiate in the ammonium ferric alum solution—controlling the results by examining the tissue with the microscope.
8. Wash in running water for 15 to 60 minutes.
9. If desired, sections may be counterstained with van Gieson's stain or eosin.
10. Differentiate and dehydrate in 95% alcohol followed by absolute alcohol.
11. Clear in xylol and mount.
12. *Results:*
    a. Chromatin, nucleoli, mitochondria, centrioles are stained black.
    b. Other tissue elements stain with the counterstain.
    c. This is a satisfactory stain for demonstrating mitotic figures, nuclear details, and protozoa.
13. *Staining Solutions.*
    a. *Ammonium ferric alum solution.*

Ferric ammonium sulfate (use violet crystals)	2.5 gm.
Water, distilled	100.0 cc.

b. *Hematoxylin solution*—1%.

Saturated hematoxylin in absolute alcohol (10%)	1 cc.
Water, distilled	99 cc.

C. *Aniline Blue Stain for Connective Tissue (Heidenhain's Modification of Mallory's Stain).*

1. Tissues fixed in Zenker's, Bouin's fluid, or formalin stain well by this method.
2. Carry paraffin sections through xylols, alcohols, and water as described under hematoxylin and eosin stain.
3. Place the deparaffinized sections in azocarmine solution in a glass-covered dish, stain in the paraffin oven at 51° to 55°C. for 45 to 60 minutes, and then cool to room temperature for 5 to 10 minutes.
4. Wash in distilled water.
5. Differentiate in an alcoholic solution of aniline.

Aniline	1 cc.
Alcohol (90%)	1000 cc.

6. Rinse in acetic acid alcohol for 30 seconds to 1 minute.

Acetic acid, glacial	1 cc.
Alcohol (95%)	100 cc.

7. Mordant in 5% aqueous solution of phosphotungstic acid for 1 to 3 hours.
8. Wash quickly in water.
9. Stain in aniline blue solution 30 minutes to 1 hour.
10. Wash quickly in water.
11. Differentiate in 95% alcohol followed by absolute alcohol.

12. Clear in xylol and mount.
13. *Results:*
    a. Collagen and reticulum stain intense blue.
    b. Chromatin, muscle tissue, and erythrocytes stain red.
    c. Neuroglia stains reddish and mucin stains blue.
14. *Staining Solutions.*
    a. *Azocarmine solution.*

Azocarmine B	0.25-1 gm.
Water, distilled	100 cc.
Acetic acid, glacial	1 cc.

1) Dissolve the azocarmine B in water and add the glacial acetic acid.
2) If azocarmine G is used, add 0.1 gm. to 100 cc. of water, bring to a boil, cool, filter, and add the glacial acetic acid.

b. *Aniline blue solution.*

Aniline blue	0.5 gm.
Orange G	2.0 gm.
Water, distilled	100.0 cc.
Acetic acid, glacial	8.0 cc.

1) Boil and filter after cooling.
2) For staining, dilute this stock solution 1 to 3 with distilled water.

D. *Van Gieson's Stain for Connective Tissue.*

1. Tissues fixed in any of the above fixatives stain well by this method.
2. Carry paraffin sections through xylols, alcohols, and water as described under hematoxylin and eosin stain.
3. Stain the deparaffinized sections for 5 to 20 minutes in Wiegert's iron hematoxylin solution.
4. Wash in water.
5. Stain for 5 to 7 minutes in van Gieson's solution.
6. Transfer directly to 95% alcohol, followed by absolute alcohol.
7. Clear in xylol and mount.
8. *Results:*
    a. Collagen stains brilliant red.
    b. Smooth and striated muscle, cornified epithelium, and neuroglia stain yellow.
    c. Nuclei stain blue.
9. *Solutions.*
    a. *Weigert's iron hematoxylin solution.*

Solution A.

Hematoxylin	1 gm.
Alcohol (95%)	100 cc.

Solution B.

Iron chloride (29% aqueous solution)	4 cc.
Water, distilled	95 cc.
Hydrochloric acid (conc.)	1 cc.

Mix equal parts of Solutions A and B fresh before using.

b. *Van Gieson's solution.*

Acid fuchsin (1% aqueous solution)	5-15 c
Picric acid, saturated aqueous solution (1.22%)	100 c

The amount of acid fuchsin added depends on the intensity of stain desired the larger amount is used for tissues fixed in Zenker's fluid and for sections of the nervous system.

E. *Weigert's Stain for Elastic Fibers.*

1. Tissues fixed in any of the above fixatives stain well by this method.
2. Carry paraffin sections through xylols, alcohols, and water as described under hematoxylin and eosin stain.
3. Stain the deparaffinized sections for 5 to 20 minutes in Weigert's iron hematoxylin solution.
4. Wash in water.
5. Stain 20 minutes to 1 hour or longer in the fuchsin solution.
6. Wash off excess stain in 95% alcohol.
7. If the sections are stained diffusely, differentiate in acid alcohol for several minutes.
8. Wash thoroughly in water.
9. Stain for 5 to 7 minutes in van Gieson's solution.
10. Transfer directly to 95% alcohol followed by absolute alcohol.
11. Clear in xylol and mount.
12. *Results:*
    a. Elastic fibers and nuclei stain dark blue to black.
    b. Collagen stains pink to red.
    c. Other tissues stain yellow.
13. *Fuchsin Solution.*

Fuchsin, basic	2 gm.
Resorcinal	4 gm.
Water, distilled	200 cc.

a. Bring the solution to a boil in an enamel dish and, when briskly boiling, add 25 cc. of a 29% aqueous solution of ferric chloride.
b. Stir and boil for 2 to 5 minutes more; a precipitate will form.
c. Cool and filter; the filtrate is discarded.
d. Leave the precipitate on the filter paper until it is thoroughly dry.
e. Return the filter paper and precipitate to the enamel dish, which should be dry but still contain whatever part of the precipitate remains adherent to it.
f. Add 200 cc. of 95% alcohol, heat carefully, and stir constantly.
g. Remove the filter paper when the precipitate is dissolved off.

h. Cool, filter, add 95% alcohol to make up to 200 cc., and then add 4 cc. of conc. hydrochloric acid.

i. The solution keeps well for months.

. *Wilder's Silver Impregnation Method for Reticulum Fibers.*

1. Tissue fixed in formalin or Zenker's fluid stains well by this method.
2. Celloidin, paraffin embedded, or frozen sections may be used.
3. Carry sections through xylols, alcohols, and from water transfer into a 10% aqueous solution of phosphomolybdic acid for 1 min.
4. Rinse in distilled water.
5. Dip in 1% aqueous solution of uranium nitrate—5 secs.
6. Wash in distilled water—10 to 20 secs.
7. Place in ammoniacal silver hydroxide solution—1 min.
8. Dip quickly in 95% alcohol.
9. Place in reducing solution—1 min.
0. Wash in distilled water.
1. Tone in 1-500 aqueous gold chloride solution—1 min.
2. Rinse in distilled water.
3. Place in 5% aqueous solution of sodium thiosulfate—1 to 2 min.
4. Wash in tap water.
5. Counterstain, if desired, with hematoxylin (Harris' solution) or van Gieson's solution.
6. Differentiate and dehydrate in 95% alcohol followed by absolute alcohol.
7. Clear in xylol and mount.
8. *Results:*
   a. Fine reticulum fibers are stained black, collagen a rose color.
   b. This is also a satisfactory stain for neurofibrils.
9. *Solutions.*
   a. *Ammoniacal silver hydroxide solution.*
      1) To 5 cc. of 10.2% aqueous solution of silver nitrate, add ammonium hydroxide drop by drop until the precipitate which forms is dissolved.
      2) Add 5 cc. of 3.1% sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonium hydroxide.
      3) Make the solution up to 50 cc. with distilled water.
   b. *Reducing solution.*

Water, distilled	50.0 cc.
Formalin, neutralized with magnesium carbonate	0.5 cc.
Uranium nitrate, 1% aqueous solution	1.5 cc.

G. *Staining Bacteria in Sections (Gram-Weigert Method).*

1. Tissue is preferably fixed in Zenker's fluid.
2. Carry sections through xylols, alcohols, water, and then stain lightly with hematoxylin solution.
3. Wash in several changes of tap water.
4. Aqueous eosin solution (2.5%)—5 min.
5. Wash in water.
6. Stain in aniline crystal violet solution—30 min.
7. Wash in water.
8. Gram's iodine solution—1 to 2 min.
9. Wash in water.
10. Blot with filter paper.
11. Decolorize in aniline-xylol (equal parts of aniline oil and xylol) until no more color comes out.
12. Clear in xylol and mount.
13. *Results:*
    a. Gram-positive organisms are deep violet; gram-negative bacteria do not stain.
    b. Nuclei stain blue to violet; connective tissue red.
14. A section of tissue known to contain bacteria should be stained simultaneously with the unknown section to serve as a control on the stain.
15. *Sterling's Crystal Violet Solution.*

Crystal violet	5 gm.
Alcohol, absolute	10 cc.
Aniline oil	2 cc.
Water	88 cc.

Solution keeps well.

H. *Stain for Tubercle Bacilli in Sections.*

1. Tissue fixed in alcohol, formalin, or Zenker's fluid stains well by this method.
2. Carry paraffin sections through xylols, alcohols, and water as described under hematoxylin and eosin stain.
3. Stain the deparaffinized sections with carbolfuchsin solution for 30 minutes in a 56°C. oven.
4. Cool in the refrigerator for 30 minutes and rinse well in tap water.
5. Decolorize in acid alcohol (1% HCl in 70% alcohol) to a faint pink.
6. Wash thoroughly in running tap water for 10 minutes.
7. Counterstain in methylene blue solution—30 sec. to 1 min.
8. Rinse in distilled water.
9. Differentiate in 95% alcohol and dehydrate in absolute alcohol.
10. Clear in xylol and mount.

11. *Results:*
    a. Tubercle bacilli stain brilliant red.
    b. Nuclei stain dark blue and other structures a lighter blue.
12. A section of tissue known to contain tubercle bacilli should be stained simultaneously with the unknown section to serve as a control on the stain.
13. *Solutions.*
    a. *Carbol-fuchsin solution.*

NaCl solution (10%)	3
Ziehl-Neelsen carbol-fuchsin stain	100

    Mix fresh before staining.
    b. *Methylene blue solution*—dilute Lo fler's methylene blue solution 1-10 w distilled water.

Basal Metabolism

Metabolism is the sum total of all the physical nd chemical processes by which living tissue is roduced and maintained. It includes the transormation by which energy for muscular acivity, production of heat, and maintenance of ital functions is made available for use by the rganism. Metabolism may be subdivided into nabolism (assimilative process) and catabolism destructive process).

Basal metabolism is the basal or minimal mount of energy (heat) produced by an inlividual measured 12 to 16 hours after eating vhen the individual is as nearly as possible at omplete mental and physical rest, but not sleep. It is the energy expended under basal onditions to maintain respiration, circulation, eristalsis, muscle tonus, body temperature, landular activity, and other vegetative funcions. Measurement of this minimal metabolism s made by means of a calorimeter—the heat roduced is expressed in large calories. A large alorie is the amount of heat necessary to raise he temperature of one liter of distilled water rom 15° to 16°C. The basal metabolism is roportional to the surface area of the body and aries with the age and sex of the individual. The basal metabolic rate (B.M.R.) is the perentage variation from the normal for an inividual of the same surface area, age, and sex.

Methods of Measuring Basal Metabolism.

Principle of Direct Calorimetry.

The rate of heat production expressed in Calories may be measured directly from a temperature change as in the determination of the heat combustion of various foods.

Respiratory chambers or especially equipped rooms may be used to measure the gaseous exchange as well as the heat produced. This method is not applicable to routine laboratory tests and is used chiefly in research work.

***Principle of Indirect Calorimetry* (Closed Method).**

Oxygen consumption can be used as an index for calculating heat production because one liter of oxygen produces a known number of Calories when it is completely used in combustion.

2. The patient breathes into and out of a container of oxygen either for a fixed length of time, during which the decrease in volume of oxygen is noted (Benedict-Roth and Sanborn apparatus), or during the absorption of a fixed volume (one liter) of oxygen, for which the time required is noted (Jones apparatus). The carbon dioxide expired by the patient is absorbed by soda lime placed in the container.
3. The rate of heat production per hour per square meter is determined from the rate of utilization of oxygen.

II. General Considerations.

A. *Patient must be in a Basal Condition.*

1. Completely relaxed, physically and mentally. (If the pulse rate increases more than a few points, it denotes some cause of disturbance.)
2. No voluntary movements during the determination.
3. Completely rested (at least 8 hours sleep).
4. No apprehension of test—the results of the first basal metabolism should be discarded and considered only as a practice test.
5. Normal temperature; the metabolic rate rises 7 per cent with each degree Fahrenheit of fever.
6. Must be in a postabsorptive state.
7. Medication.
   a. No thyroid extract for at least 1 to 3 weeks depending on the dose.
   b. No caffeine, adrenalin, or sedatives for at least 24 hours.

B. *Care of Machine for Indirect Calorimetry* (see Fig. 33).

1. Soda lime must be changed after 35 to 40 tests.
   a. Progressive increase in respirations with lengthening of each successive breath indicates an exhausted soda lime supply.
   b. To test the soda lime, allow some of the oxygen left in the tank to bubble through a solution of 10% barium hydroxide; if there is any CO_2 present, the solution will become cloudy.
2. The flutter valves must be changed when they lose their elasticity and the lips fail to close properly.

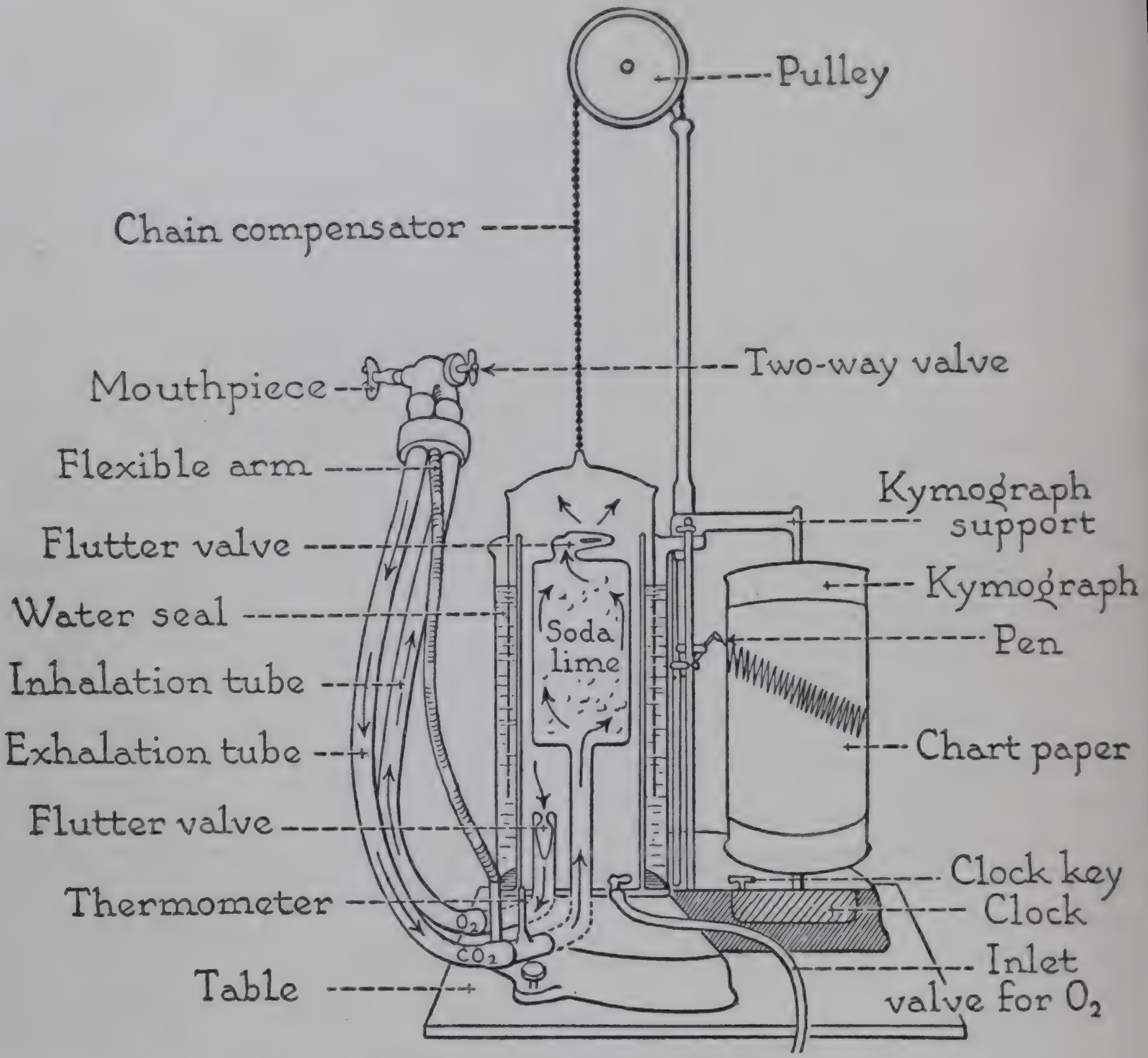
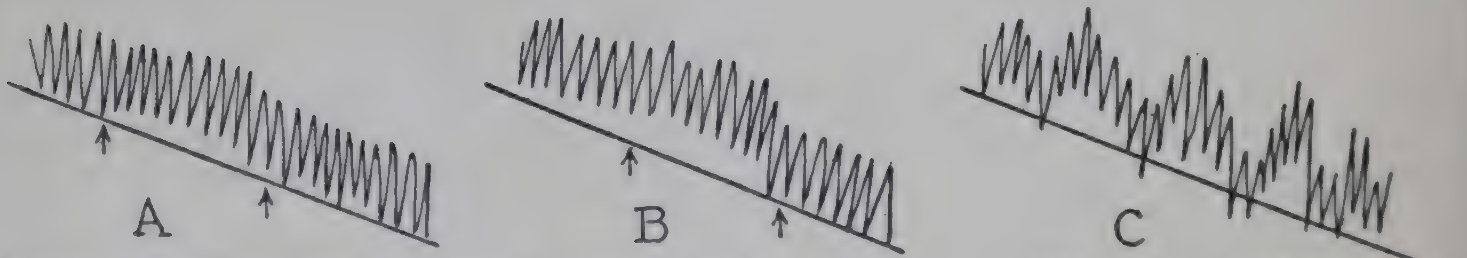

Pulley
Chain compensator
Two-way valve
Mouthpiece
Flexible arm
Kymograph support
Flutter valve
Kymograph
Water seal
Soda lime
Pen
Inhalation tube
Exhalation tube
Chart paper
Flutter valve
Clock key
Thermometer
Clock
O_2
CO_2
Inlet valve for O_2
Table

DIAGRAM of BENEDICT-ROTH BASAL METABOLIC MACHINE



A Normal tracing

B Tracing showing leak in machine

C Tracing showing abnormal breathing — not basal

Fig. 33. Basal Metabolic Machine and Sample Tracings. The arrows in the tracings indicate the placing and removal of the weight in testing for a leak in the machine.

Test for an oxygen leak in the machine.

a. Place recording paper around the kymograph drum.

b. Fill the spirometer chamber with oxygen and mark its height on the recording paper with the pen.

c. See that all connections are turned tightly.

d. Place testing weight on bell and leave overnight.

e. If there is a leakage of oxygen, the bell will be lower than when filled and the pen will be at a higher level than the original mark.

The clock should be checked occasionally with a stop watch.

Change oxygen tank when empty.

III. Instructions for Patient.

Out Patient.

Eat a light supper (very little protein) not later than 7 o'clock the night before the test.

Go to bed early (by 10 o'clock at least).

Upon arising in the morning, dress slowly; do not rush.

Do not eat *any* breakfast; this means nothing to *eat* or *drink* except water.

Do not smoke.

Come to the hospital with as little exertion as possible.

Hospital Patient.

Should have a light supper the night before the determination.

Given no breakfast.

If in a private room, the test is taken at the bedside as soon as possible after the patient awakens.

If in a ward, the patient is taken to the metabolism room in a wheel chair and must rest for 30 minutes after being put to bed.

IV. The Test.

Machine Before Test.

Wrap recording paper tightly around kymograph drum and paste in place with gummed paper.

Fill cup of recording pen with ink.

Wind clock.

Test water level in spirometer; should be 3 inches from top.

By means of a rubber tube, connect the oxygen compression cylinder to the pet cock on the machine for introducing oxygen gas.

Admit oxygen into the spirometer chamber until the pen comes within 2 inches of the bottom of the paper. Close valve on gas tank and pet cock on machine.

7. Attach sterile mouthpiece to machine.
8. Have the following ready: nose clip, towel, and weight for testing the machine.

B. *Patient.*

1. If an outpatient, ascertain whether he has carried out instructions.
2. Take the patient's pulse and temperature; if he has any fever, take test on another day.
3. Have patient empty bladder so there is no discomfort from distention.
4. If patient is fully clothed, have him remove shoes and take off or loosen any clothing that binds the body (girdles, belts, collars, etc.).
5. He must rest quietly in bed for 30 minutes immediately preceding the test.
6. The room should be quiet, have subdued lighting and plenty of fresh air and the patient must be warmly covered.
7. Remove any apprehension of the test from the patient's mind and assure him that there will be no difficulty in breathing.
8. Instruct the patient to lie quietly in a comfortable position, to close his eyes, to breathe regularly and naturally, and not to think of the test.
9. Explain that the mouthpiece should be inserted so that the flange is between the gums and the lips and the tabs between the upper and lower teeth; the valve on the machine should be turned so the patient will be breathing room air.
10. Apply the nose clip so that the sponge rubber pads are well down on the nose, press them together firmly but gently, and adjust the thumb screw to hold the nose clip in this position.
11. Ask the patient to try to exhale through his nose; if he can, the nose clip is not tight enough and should be readjusted until this is impossible.

C. *Test.*

1. Close the valve on the machine so the patient is breathing oxygen from the spirometer chamber.
2. Start the kymograph revolving.
3. Record the temperature of the gas in the machine.
4. Take the patient's pulse.
5. Allow test to run 6 minutes.
    a. After 3 minutes, place a leak tester weight (about 50 gm.) on top of the bell and leave it there until the pen crosses the next line.

   b. In case there is a slight leak around the nose clip, mouthpiece, or breathing tube connections, this added weight on top of the bell will cause the oxygen to escape faster and the respirations will assume a steeper angle (see Fig. 33).
   c. If there is a leakage, readjust mouthpiece and nose clip.
6. Refill spirometer with oxygen and repeat test for another 6 minutes.
7. Remove nose clip and mouthpiece.
8. Record temperature of gas in the machine.
9. Take patient's pulse.
10. Obtain patient's height (stocking feet) and weight, subtracting for clothes up to 2 lbs. depending on amount of clothing.
11. Record age and sex of patient.

D. *Machine After Test.*

1. Disconnect breathing tubes so any moisture that may have collected in them will drain out.
2. Place wire in pen point and a small strip of blotter in ink cup.
3. Cover machine.
4. Wash mouthpiece and nose clip with soap and water.
5. Sterilize mouthpiece by boiling in water for 3 minutes.

V. Calculation.

A. *Calories of Heat Produced per Hour.*

1. Using the ruler, draw the oxygen consumption line.
   a. Select the portion of the graph which presents the most uniform trend and draw the line according to the general slope.
   b. This line should be close to but preferably under the expiration points, avoiding intersection of the deeper ones.
2. Draw a similar line for the second 6 minutes graph; this should be practically parallel to that of the first 6 minute period.
3. Measure in millimeters the rise in height of the oxygen consumption lines during each 6 minutes. (For each degree rise in temperature in the spirometer bell, 0.5 mm. must be added to the rise in consumption line.)
4. The two lines should check within 5 per cent.
5. Determine the volume of oxygen used in cc. by multiplying the rise in millimeters by 20.73 cc. This is the number of cc. in a base area of the spirometer 1 mm. in height, a constant for the Benedict-Roth machine.
6. Make calculations for both oxygen c
   sumption lines.
7. Correct for barometric pressure (B.P
   temperature in degrees centigrade (T), a
   moisture by using a chart or by the f
   lowing formula:

$$\text{Factor} = 0.0012 \times \text{B.P.} + 0.001\ [4.6\ (30 - \text{T}) -$$

8. Determine cc. of oxygen absorbed for o
   hour by multiplying the amount obtain
   in 6 minutes by 10 (all previous calcul
   tions were for 6 minutes).
9. Calculate the number of Calories of he
   by multiplying the number of liters of ox
   gen per hour by 4.825 (cc. of oxygen mu
   be changed to liters by dividing by 1000
10. The Benedict-Roth machine is so made th
    the area of the base of its spirometer be
    when multiplied by 10, divided by 100
    and multiplied by 4.825 equals 1. There
    fore, one millimeter rise in the oxygen con
    sumption line is equal to one Calorie o
    heat. Thus, the calculation is reduced t
    multiplying the height of the oxygen con
    sumption line by the correction factor fo
    the barometric pressure and temperature.

$$\frac{A \times 20.73 \times B \times 10 \times 4.825}{1000} = A \times B$$

A = rise of oxygen consumption line in mm.
B = correction factor for barometric pressure
temperature, and moisture.

B. *Calories According to Surface Area.*

1. *Surface Area in Square Meters.*
   a. Using the height and weight, obtain the
      surface area from a standard chart.
   b. If a chart is not available, DuBois' formula
      can be used.

$$\text{S.A.} = \text{Wt.}^{0.425} \times \text{Ht.}^{0.725} \times 71.84$$

S.A. = surface area in sq. cm.
Wt. = weight in kg.
Ht. = height in cm.

2. The Calories per square meter of surface
   area is obtained by dividing the Calories per
   hour by the surface area.
3. Using formula in A-10.

$$\frac{A \times B}{\text{S.A.}} = \text{Calories per sq. m. per hr.}$$

C. *Basal Metabolic Rate.*

1. Obtain the standard Calories per square meter per hour for a normal person of the
   same sex and age from a standard chart
   (Aub-DuBois, Boothby and Sandiford, Harris-Benedict, Benedict-Talbot, or Benedict-Hindry-Baker).
2. Calculate the percentage above or below
   normal by the following formula:

$$\frac{\text{Patient's Calories} - \text{Standard} \times 100}{\text{Standard}} = \text{B. M. R.}$$

VI. Possible Errors.

Patient may have difficulty in breathing because of the following:

Moist soda lime.
Water in base of machine or in breathing tubes.
Flutter valve stuck together.

A False Low B.M.R. may be due to:

Oxygen leaking into spirometer through oxygen pet cock.
Inefficient soda lime.
Clock running too fast.

A False High B.M.R. may be due to:

Leaks around nose clip or mouthpiece.
Leakage around attachment of rubber tubing.
Clock running too slow.

VII. Interpretation.

Normal B.M.R.: ± 15 per cent.

Factors Influencing Basal Metabolism.

Age and sex
Surface area
Athletic training
Climate and altitude
Sleep

Decreased Metabolism found in:

Starvation and undernutrition
Obesity due to pituitary or hypothalmic disorders.
Hypothyroidism
Hypopituitarism
Simmonds disease (—40%)
Addison's disease (—20%)
Lipoid nephrosis
Shock
Arterial hypotension

Increased Metabolism found in:

Hyperthyroidism (exophthalmic and toxic goiter)
Fever
Diabetes insipidus
Cardiorenal disease with dyspnea (+25 to +50%)
Leukemia (+20 to +80%)
Polycythemia (+10 to +40%)
Acromegaly (early stage)
Pituitary basophilism (Cushing's disease)
Hypercorticoadrenalism
Essential hypertension
Severe anemia
Sprue
Osteitis deformans (Paget's disease)

E. *Causes of False Increase in the B.M.R.*

Shivering, tremors, exertion during test.
Digestion and absorption of food before test.
Smoking
Mental state of patient
Premenstrual increase
Pregnancy after 5 months
Pain and discomfort
Fever
Labored breathing or excessive pulmonary ventilation.
Increase in pulse rate

Vital Capacity

I. Method (Benedict-Roth Metabolism Machine).

A. *Machine.*

1. The spirometer bell must be at its lowest point.
2. Place chart on kymograph so the pen will touch the top of the vital capacity section.
3. Fill the pen with ink.
4. Open the two-way breathing valve at the mouthpiece so that it is connected to the machine—not to room air.
5. Raise the flexible arm with the breathing tubes and valve to a height so that the open end is directly in front of his mouth when the patient is standing in front of the machine.
6. Connect the short rubber tubing to the open end of the two-way valve and insert the vital capacity mouthpiece into the other end.

B. *Patient.*

1. With the patient standing, have him take a full breath, filling the lungs to the utmost, close off the nostrils with the thumb and forefinger, and exhale into the machine.
2. The pen will make a straight line downward as the bell rises to the height of the exhalation.
3. After the exhalation peak has been reached, the bell will sink and the pen return to its starting point.
4. Take 2 or 3 readings.

C. *Calculation.*

1. Read the vital capacity directly from the chart, reporting the reading showing the greatest capacity.
2. Obtain the patient's normal vital capacity by multiplying his body surface in square meters by 2500 cc. for men and 2000 cc. for women.
3. Calculate the per cent of normal.

$$\frac{\text{Patient's Capacity}}{\text{Normal Capacity}} \times 100 = \text{per cent of normal.}$$

II. Decreased Vital Capacity due to the following:

A. *Conditions Directly Involving the Lungs.*

Pneumonia
Pulmonary tuberculosis
Emphysema
Silicosis

B. *Mechanical Interference with Expansion of Thoracic Cavity.*

Abnormalities of thoracic walls
Restriction by pain
Interference of movement of the diaph
by abdominal condition.

C. *Intrathoracic Conditions.*

Pleural effusion
Pericardial effusion
Pneumothorax
Tumors

D. *Heart Disease.*

Pulmonary edema
Passive congestion of the lungs

Electrocardiography

The electrocardiogram is a picture that ex- esses certain electrophysiologic phenomena nifested by the heart during the process of ntraction. When the heart muscle contracts, e active part becomes electronegative to the ctive part. In this way an electromotive force set up with positive and negative potential ferences that are led off and are recorded an electrocardiograph machine. When the tire heart becomes active, both poles become gatively charged and no flow of current takes ce in the outside circuit. The muscle is then an isoelectric state, but negatively charged.

I. Electrocardiograph Machine (Cambridge Type).

Principle: The flow of current from the heart is conducted through a galvanometer string inducing lateral deflections in it. These deflections appear as a continuous shadow on photographic sensitive paper which is moving at a desired speed. They are magnified 600 diameters and brought to focus by an optical system consisting of an arc light, a condenser, and a microscopic lens (see Fig. 34).

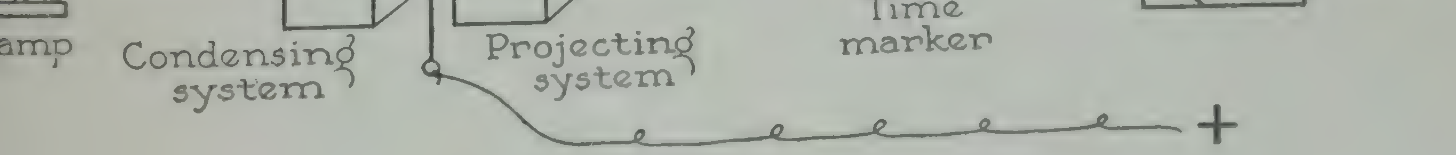


Fig. 34. Diagram of the Principal Parts of a String Electrocardiograph Machine.

Galvanometer String.

It is a straight conducting strand lying vertically between the two poles of a powerful magnet.

It is a finely drawn quartz thread, from 0.002 to 0.005 mm. in diameter, coated thinly by a film of platinum, gold, or silver.

3. The shadow of the string moves from side to side leaving upon the developed film a white line that varies in thickness according to the rate at which the string is moving.
4. Due to the extreme fragility of the string, it is kept taut and protected whenever the machine is not in use.

C. *Galvanometer Lamp and Condenser.*

1. The lamp has a special straight line filament, rated at 0.5 ampere, 4 volts.
2. When a new lamp is inserted into the machine, the condenser tube must be adjusted so that the image of the filament just fills the field of light.
3. The rear focusing nut must be adjusted until satisfactory definition of the picture is obtained.
    a. Too little "backoff" will give pictures which have a black background, but the string shadow is indistinct and the time lines appear to show through the string.
    b. Too much "backoff" gives pictures in which the string shadow may be good, but the background is too pale.

D. *Microscopic Lens.*

1. The cylindrical lens brings the light to a focus.
2. It is ruled in millimeter spaces and these lines are photographed on the film as it travels making the horizontal lines on the film.

3. The space between each horizontal line (1 mm.) is equivalent to 0.1 millivolt of current after proper standardization while the space between every fifth horizontal (heavy) line (5 mm.) represents a deflection of 0.5 millivolt standard current.
4. Each electrocardiogram is standardized so that one millivolt of current causes a 10 mm. (1 cm.) excursion of the string.
    a. If it is less, the string must be slackened.
    b. If it is more, the string must be tightened.
    c. The deflection must be accomplished in 0.02 seconds or less and there must be no overshooting before it reaches a new level.
    d. A standardization must be done for each lead.
5. This standardization permits ready comparison of electrocardiograms from different instruments.

E. *Time Marker.*

1. A toothed wheel driven by an electrical current and controlled by a timing fork revolves and cuts the beam of light at regular intervals of one-twenty-fifth of a second, making the vertical white lines on the film. Every fifth tooth on the wheel is larger than the others and makes a wider white line on the film.
2. On the electrocardiogram the space between each vertical line represents 0.04 seconds, while that between every fifth vertical line (heavy) represents 0.20 seconds.

F. *Battery.*

1. The battery used in a large machine is an Everready No. 950, while a No. 935 is used in a portable machine.
2. It brings the standardizing current to 1 milliampere.
3. The current must be reversed when the string cannot be brought back to the center of the field.

G. *Camera.*

1. The upper film box contains the photographic paper or film. It has an automatic shutter which prevents the exposed paper from being light struck through the narrow aperture.
2. To refill, remove the box from the machine and place the roll of paper in the box in a dark room. The film for a portable machine need not be changed in a dark room.
3. The camera motor drives the photographic paper past the aperture at about 12.5 revolutions per minute. The camera motor r independently of the time marker.
4. A white mark is painted on the edge of camera pulley for convenience in noting revolutions.
5. The lower film box catches the expo paper.

H. *Electrodes.*

1. The electrodes are made of German sil and are attached to the patient at speci locations, both wrists and the left ank
2. The exploring electrode used in taking t precordial leads (Lead IV) should be a c cular disc less than 3 cm. in diameter.
3. Because the electrodes are not in direct co tact with the heart itself, they are call "indirect leads"; however, when one ele trode is placed over the heart (precordi leads), they are called "semidirect leads."

II. Care of the Machine.

1. When not in use, the main switch shoul be kept at the "off" position, the electrod cable in place, and the cover over the to of the machine.
2. Remove the electrical connection and groun wire.
3. The electrodes should be kept clean at al times and sandpapered occasionally.
4. The battery should be checked periodically with the voltmeter using the selector switch.
5. The film box should always be in the machine except when developing films.

III. Method of Taking an Electrocardiogram.

A. *Machine.*

1. Connect power supply cable to wall socket.
2. Attach grounding wire to a clean metallic surface free from paint or corrosion on a cold water pipe or radiator.
3. All electrical apparatus in the room should be disconnected, such as lamps, radios, fans, etc.

B. *Applying Electrodes to Patient.*

1. The patient should be completely relaxed and at ease, preferably lying down.
    a. It is important to designate the position of the patient while taking a particular electrocardiogram.
    b. Follow up grams should be made in the same position.
2. Squeeze one-half inch of lubricating jelly onto the skin of the outer or inner forearms, the outer or inner left leg just above the ankle (not over the bone), and on the chest over the heart.

Rub the jelly briskly into the skin with a tongue depressor covering an area slightly greater than the electrode (3″x 2″) until a slight erythema is produced. If the skin is very hairy, oily, or hard, massage longer than usual.

Immediately strap one electrode on each arm and on the left leg.

- a. They should be just tight enough so that the electrode does not slide over the skin when moved gently by hand.
- b. Do not strap too tightly or they may produce muscle tremor in the electrocardiogram.

The patient holds the exploring (precordial) electrode with one hand by placing the index finger on the top of the handle, sufficient pressure is exerted to hold the electrode securely to the chest.

If the electrodes are applied correctly, the skin resistance should not exceed 2000 ohms. In case higher resistance is obtained or if the string shows a tendency to wander, remove the electrodes, wipe skin clean, and reapply the lubricating jelly.

After taking tracings, remove the electrode jelly from the skin with a damp sponge or cloth, rinse electrodes in warm water, and dry thoroughly.

Operation of the Machine.

Connect the lead wires to the electrodes on the patient.

- a. The red lead tip goes to the right arm.
- b. The white to the left leg.
- c. The black to the left arm.
- d. The brown to the exploring electrode.

The lead selector and compensator knob should be set at "0."

Turn the electricity on by turning the main switch to "on."

Center the string by means of the centering lever.

Focus the string by turning the focusing nut until the string shadow appears dense with very narrow bright bands along both edges.

Standardize the string so that its shadow moves just 3 cm. when 3 millivolts of current are introduced into the circuit from the battery. If adjustment is necessary, turn wheel on the suspension cover plate until the correct deflection is obtained.

Make connection between Lead I and the galvanometer.

- a. The current from the body is not thrown into the circuit in full force at one time but through several levels of extra resistance by shunts.
    - 1) At each stage neutralization of the body current is accomplished by moving the compensator so that the string is recentered.
    - 2) On the portable machine there is only one shunt, while larger machines have two (first 1:100, then 1:10).
- b. This is to prevent a relatively strong current from causing a sudden wide sharp deflection of the galvanometer string which might cause it to break or its metallic coating to become separated.

8. Standardize the string again so that one millivolt of current causes a 1 cm. excursion of the string when the selector switch is in position "I" (never standardize the string before "I" is reached).
9. Turn the camera on and number the film.
10. Allow the film to run about 15 seconds; during this time record the sensitivity of the string by applying the standardizing impulse of one millivolt.
11. Return the compensator knob to zero.
12. Repeat procedure from 7 for lead II, adjusting the selector switch to position "II."
13. Repeat again for lead III, adjusting the selector switch to position III.
14. Repeat again for lead IV-F.
    - a. The patient holds the exploring electrode in position on the chest (see III B).
    - b. The selector switch is adjusted to position "IV."
    - c. If the machine has only 3 lead wires, remove the lead wire from the left leg and attach to the exploring electrode, then place the lead wire from the right arm to the electrode on the left leg. The selector switch is adjusted to position "II."
15. When all the leads desired are recorded, cut the film.
16. Turn the lead selector and compensator to "0."
17. Tighten the string to one-third normal sensitivity by pressing the standardizing level to 3 millivolts position and turning the string tension adjusting wheel to the left until the string deflects about 1 cm. for 3 millivolts.
18. Turn the switch to the machine to "off."
19. Remove the electrodes from the patient.

D. *Developing the Film.*

1. Remove film box and take to the dark room.
2. Take out film when room is dark and place in water until completely wet.

3. Place in developer for 2 to 4 minutes depending on age and temperature of the developer.
4. Wash in water and place in hypo solution for 10 to 20 minutes or until the film becomes completely fixed.
5. Wash in running water for 10 to 30 minutes.
6. Roll out on chromium or ferrotype boards with exposed side down to dry. This prevents the film from curling.

E. *Cutting Film to Place in Mounts.*

1. Cut film to fit mounts.
2. Include one standardization in each lead.
3. Include any abnormal beats, such as ectopic, even if one must exclude the standardization which can be added separately, but do not include artifacts.

F. *Artifacts*—bizarre and irregular deflections in the tracing which are due to mechanical defects of the instrument (extrinsic) or muscular movements of the patient (intrinsic origin).

1. *Intrinsic Origin.*
   a. Muscular tension, tremor, or tic.
   b. Voluntary muscular contractions.
   c. High skin resistance.
2. *Extrinsic Origin.*
   a. High resistance of electrodes.
   b. Varying resistance with polarity.
   c. Loose or unclean contacts.
   d. Extraneous currents.
   e. Improper neutralization of skin current.
   f. Inaccurate standardization.
   g. Too loose string.
   h. Poor illumination.

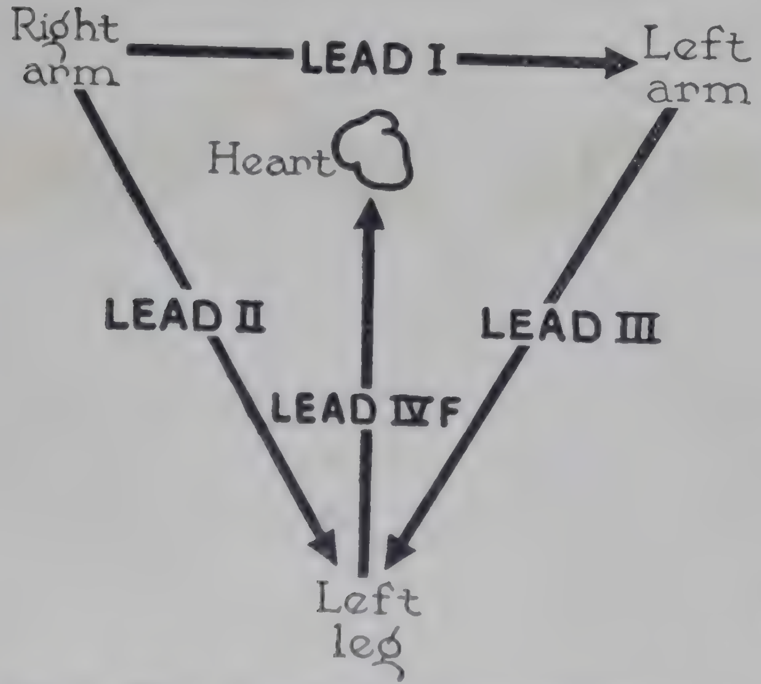


Fig. 35. Place of application of the electrodes in the different leads.

   i. Faulty action of time marker or
   j. Defective development or pri record.

IV. Relation of the Leads to the H

A. *Indirect Leads* record the action heart muscle by placing the electr the extremities (see Fig. 35).

1. Lead I is from the right arm to the l across the base of the heart.
2. Lead II is transversely across the hea the right arm to the left leg.
3. Lead III is along the left border of th from the left arm to the left leg.

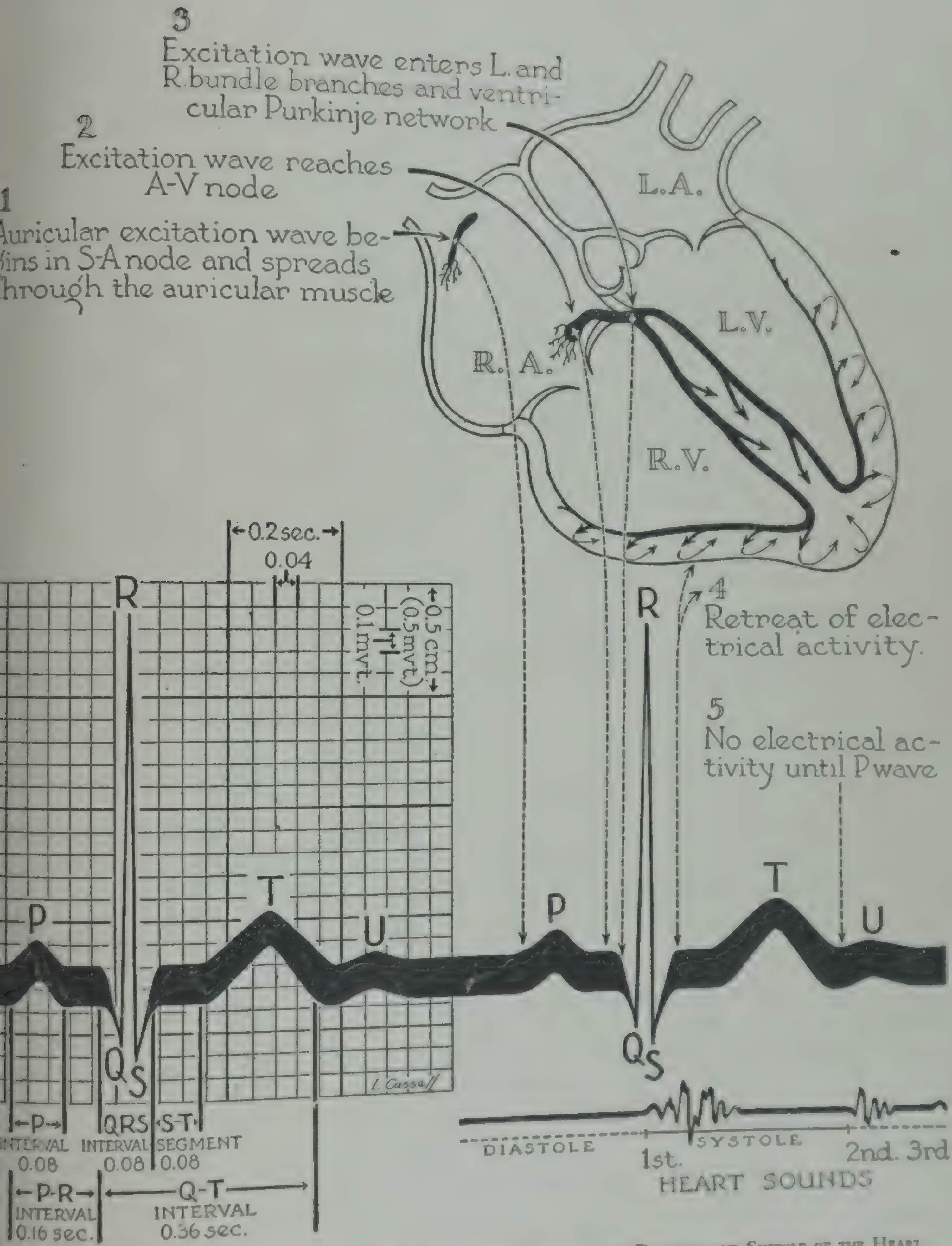
B. *Semidirect (Precordial) Leads.*

1. These are made by placing the ex electrode over the precordial region.
2. The indifferent electrode is on the l (IV-F); see Fig. 35.
3. If only one fourth lead is taken, th cordial electrode is placed at the e outer border of the apex beat. If th beat cannot be located, the electrode is in the fifth intercostal space lateral t left midclavicular line.
4. If all six precordial leads are taken, th ploring electrode is placed in the foll positions:
   a. Right margin of the sternum (C1).
   b. Left margin of the sternum (C2).
   c. Line midway between the left marg the sternum and the left midclav line (C3).
   d. Left midclavicular line (C4).
   e. Left anterior axillary line (C5).
   f. Left midaxillary line (C6).

V. Interpretation of a Normal Electrocardiogram.

A. *Correlation of the Electrocardiogram the Action of the Heart* (See Fig. 36)

1. The P wave is due to auricular contra
2. The P-Q (or P-R) interval represents required for the electrical impulse to from the sino-auricular node through auricles, the auriculo-ventricular node, the conduction apparatus down to the t nal arborizations.
3. The QRS complex begins with the ea invasion of the ventricular myocardiur the impulse and ends when every part o ventricular muscle has been completely volved.
4. The S-T (or R-T) segment represent period of electrical activity of the heart the entire ventricular musculature has fully invaded by the electrical impulse.


3
Excitation wave enters L. and R. bundle branches and ventricular Purkinje network
2
Excitation wave reaches A-V node
1
uricular excitation wave be-
ins in S-A node and spreads
hrough the auricular muscle
L.A.
L.V.
R. A.
R.V.
4
Retreat of electrical activity.
5
No electrical activity until P wave
←0.2 sec.→
0.04
←0.5 cm.→
(0.5 mvt.)
0.1 mvt.
R
P
T
U
Q
S
P
R
T
U
Q
S
←P→
NTERVAL
0.08
QRS
INTERVAL
0.08
S-T
SEGMENT
0.08
←P-R→
INTERVAL
0.16 sec.
←Q-T→
INTERVAL
0.36 sec.
DIASTOLE
SYSTOLE
1st.
2nd. 3rd
HEART SOUNDS
I. Cassell

G. 36. RELATION OF THE ELECTROCARDIOGRAM TO THE EXCITATION WAVE AND TO DIASTOLE AND SYSTOLE OF THE HEART.

5. The T wave represents the recession of the electrical impulse in the ventricles.
6. The Q-T interval represents the entire time required for depolarization and repolarization of the ventricular musculature.
7. Occasionally U waves are seen after the T waves; their origin is still debatable.

B. ***Routine for Reading Electrocardiograms.***

1. Determine the rate of the heart.
    a. Count number of cycles in 6 inches of the electrocardiogram.
    b. Multiply by 10 to obtain rate per minute.
2. Determine the axis deviation present.
    a. If the algebraic sum of the positive (above isoelectric line) and negative (below) deflections of the QRS complex (measured in mm.) is positive in each of the first 3 leads, or if it is zero in the first or third lead and the other 2 leads are positive, electrical axis is normal.
    b. If the algebraic sum is positive in the f lead and negative in the third lead or in second and third leads, it indicates axis deviation.
    c. If the algebraic sum is negative in the fi lead and positive in the second and th leads, or negative in the first and seco leads and positive in the third, the traci is that of right axis deviation.
3. Observe any irregularities of rhythm.
4. Look for abnormalities of the P wave.
5. Determine the P-R interval (normal 0.12 0.20 seconds).
6. Look for abnormalities of the QRS comple
7. Determine the QRS interval (normal 0.06 0.10 seconds).
8. Observe any abnormalities of the T wave an the ST interval (normal 0 to 0.15 seconds

Solutions Used in Routine Tests

I. Antiseptic Wash.

Formalin	4 cc.
Ethyl Alcohol (70%)	1000 cc.

II. Preparation of Percentage Solutions of Alcohol.

A. ***To prepare from 95% alcohol:***

x = desired amount of diluted alcohol.
y = desired percentage (expressed as a decimal).

$$\frac{xy}{0.95} = \text{amount of 95\% alcohol required.}$$

B. ***Example:*** Prepare 500 cc. of 70% alcohol.

$$\frac{500 \times 0.7}{0.95} = 368.4 \text{ cc. of 95\% alcohol plus } 131.6 \text{ cc. of distilled water.}$$

III. Iodine Solution for Disinfecting Purposes.

odine Crystals	3.5 gm.
Potassium iodide	2.5 gm.
Distilled water	27.0 cc.
Ethyl Alcohol (95%)	73.0 cc.

IV. Cleaning Solution.

A. Dissolve 100 gm. of technical sodium dichromate in 100 cc. of water with the aid of heat.
B. Add 500 cc. of technical sulfuric acid to 500 cc. of water and add the dissolved dichromate to this hot solution.

V. Physiologic Salt Solution.

odium chloride, c.p.	8.5 gm.
Distilled water to make 1 liter.	

Solutions for Urinalysis

I. Benedict's Reagents.

A. ***Qualitative Solution.***

Copper sulfate (pure crystalline, $CuSO_4 \cdot 5H_2O$)	17.3 gm.
Sodium citrate, c.p.	173.0 gm.
Sodium carbonate (anhydrous) (or 200 gm. of crystalline, $Na_2CO_3 \cdot 10H_2O$)	100.0 gm.
Distilled water to make 1 liter.	

1. Dissolve the citrate and carbonate in about 600 cc. of water with the aid of heat and filter.
2. Dissolve the copper sulfate in 100 cc. of water with heat and pour slowly into the first solution, stirring constantly.
3. Cool and make up to 1 liter.
4. This reagent keeps indefinitely.
5. It can not be used for quantitative tests.

B. ***Quantitative Solution.***

Copper sulfate (pure crystalline, $CuSO_4 \cdot 5H_2O$)	18.0 gm.
Sodium carbonate (anhydrous) (or 200 gm. of crystalline, $Na_2CO_3 \cdot 10H_2O$)	100.0 gm.
Sodium citrate, c.p.	200.0 gm.
Potassium sulfocyanate, c.p.	125.0 gm.
Potassium ferrocyanide solution (5%)	5.0 cc.
Distilled water to make 1 liter.	

1. With the aid of heat dissolve the carbonate, citrate, and sulfocyanate in about 600 cc. of water and filter.
2. Dissolve the copper sulfate in 100 cc. of water with heat and pour slowly into the other fluid, stirring constantly.
3. Add the ferrocyanide solution, cool, and dilute to 1 liter.
4. The copper sulfate should be weighed on an analytical balance.
5. The solution keeps well.
6. This solution should be checked by titrating with the 1% stock standard glucose solution used for blood sugar determinations: 25 cc. of Benedict's reagent should reduce 5 cc. of the standard glucose solution.

II. Ehrlich's Reagent (Modified).

Distilled water	100.0 cc.
Hydrochloric acid (conc.)	150.0 cc.
Para-dimethylaminobenzaldehyde	0.7 gm.

III. Exton's Qualitative Reagent.

Sodium sulfate, anhydrous (or 200 gm. of $Na_2SO_4 \cdot 10H_2O$)	88 gm.
Sulfosalicylic acid	50 gm.
Distilled water to make 1 liter.	

Dissolve the sodium sulfate in 800 cc. of water with the aid of heat. Cool, add the sulfosalicylic acid, and make up to volume with water.

IV. Fouchet's Reagent.

Trichloroacetic acid	25 gm.
Distilled water	100 cc.
Ferric chloride (10%)	10 cc.

V. Obermayer's Reagent.

Hydrochloric acid (conc.)	1000 cc.
Ferric chloride	2 gm.

VI. Robert's Reagent.

Saturated magnesium sulfate (U. S. P. $MgSO_4 \cdot 7H_2O$) sol. (add 1 liter of water to approx. 800 gm.)	5 parts
Nitric acid (conc.)	1 part

VII. Sodium Nitroprusside Reagent.

Sodium nitroprusside (nitroferricyanide) crystals	10 gm.
Sulfuric acid (conc.)	2 cc.
Distilled water to make	100 cc.

Keep in a brown bottle.

VIII. Tsuchiya's Reagent.

Phosphotungstic acid (crystals)	1.5 gm.
Ethyl alcohol (95%)	93.5 cc.
Hydrochloric acid (conc.)	5.0 cc.

Dissolve the phosphotungstic acid in the alcohol before adding the hydrochloric acid.

Solutions for Hematology

I. Anticoagulant Solution.

Ammonium oxalate, c.p.	12 gm.
Potassium oxalate, c.p.	8 gm.
Distilled water to make 1 liter.	

A. Pipette 0.5 cc. of the solution into a one-half ounce bottle and warm (not over 70°C.) until the water is completely evaporated.

B. This amount prevents 5 cc. of blood from clotting. If more or less blood is used, it is important to use the anticoagulant solution in this same proportion.

II. Buffer Solution (for Wright's Stain).

Potassium phosphate, monobasic (Sørensen's KH_2PO_4)	6.63 gm.
Sodium phosphate, dibasic (anhydrous Na_2HPO_4)	2.56 gm.
Distilled water to make 1 liter.	

The pH should be 6.4.

III. Diluting Fluid for Leukocytes.

A. *Blood* (Leukocyte Count).

Hydrochloric acid	5.0 cc.
Distilled water	495.0 cc.

Add 2 small crystals of thymol as a preservative.

B. *Spinal Fluid* (Leukocyte Count).

Crystal violet	0.1 gm.
Glacial acetic acid	10.0 cc.
Distilled water	90.0 cc.

Filter; should be free of any precipitate.

IV. Diluting Fluid for Erythrocytes.

A. *Gower's Solution.*

Sodium sulfate, c.p. (anhydrous)	12.5 gm.
Glacial acetic acid	33.3 cc.
Distilled water	200.0 cc.

B. *Hayem's Solution.*

Mercuric chloride, c.p.	1.0 gm.
Sodium sulfate (anhydrous) (or 10 gm. crystalline $Na_2SO_4 \cdot 10H_2O$)	4.4 gm.
Sodium chloride, c.p.	2.0 gm.
Distilled water	400.0 cc.

V. Platelet Solution.

Sodium citrate, c.p.	1.5 gm.
Distilled water	50.0 cc.
Formalin, neutral to litmus	2.5 cc.

Keep in the refrigerator.

To neutralize acid formalin, add enough calcium carbonate to form a layer on the bottom of the bottle and let stand several days.

VI. Stains.

A. *Manson's Stain* (Modified).

1. *Stock Solution.*

Borax (sodium borate)	2.5 gm.
Boiling distilled water	50.0 cc.
Methylene blue	1.0 gm.

   a. Dissolve sodium borate in the boiling distilled water, add the methylene blue, and filter.
   b. Keeps 2 weeks.

2. *Dilute Solution.*
   a. Add 1 cc. of stock solution to 20 cc. of 0.3% NaCl solution.
   b. Must be made fresh just before using.

B. *Reticulocyte Stains.*

1. *Saline Stain.*

Brilliant cresyl blue	1.0 gm.
NaCl solution (0.85%)	100.0 cc.

Warm slightly to dissolve stain and filter when cool.

2. *Alcoholic Stain.*

Brilliant cresyl blue	1.0 gm.
Methyl alcohol	100.0 cc.

Filter before using.

C. *Washburn's Stain.*

Benzidine base	0.3 gm.
Basic fuchsin	0.3 gm.
Sodium nitroprusside (sat. aq. sol., 5 gm. in 5 cc.)	1.0 cc.
Ethyl alcohol (95%)	100.0 cc.

1. Dissolve the benzidine and fuchsin in the alcohol in the order named.
2. Add the nitroprusside solution.
3. A slight precipitate may form at the bottom of the flask but does not interfere with the staining qualities.
4. This solution will keep 1 year.

D. *Wright's Stain.*

1. All containers must be absolutely dry before using.
2. Place 0.3 gm. of powdered Wright's stain in a mortar and add 3 cc. of glycerin.
3. Grind thoroughly with a pestle.
4. Add small portions of 100 cc. of acetone-free methyl alcohol.
5. Stopper bottle tightly and let stand with occasional shaking for at least 1 week.
6. Filter before using.

Normal Solutions

I. Oxalic Acid.

Normal Solution.

Weigh out exactly 45 gm. of pure anhydrous oxalic acid ($H_2C_2O_4$) or 63 gm. of $H_2C_2O_4 \cdot 2H_2O$ and place in a liter volumetric flask.

Dissolve in about 700 cc. of distilled water and then dilute to volume at 20°C.

Invert 100 times and let stand overnight before using.

Standardization.

If the oxalic acid is accurately weighed, this solution should be exactly normal.

It can be titrated with exactly N NaOH using phenolphthalein as an indicator.

II. Hydrochloric Acid.

Normal Solution.

Add approximately 90 cc. of conc. HCl (sp. gr. 1.19, 37%) to about 700 cc. of distilled water in a liter volumetric flask.

$$\text{The cc. of HCl used} = \frac{36.5 \text{ (eq. wt.)}}{1.19 \times 0.37}$$

Allow the solution to cool and then dilute to volume with distilled water at 20°C.

Invert 100 times and let stand overnight.

Standardization.

Place 10 cc. of the above solution in an Erlenmeyer flask and add 2 drops of phenolphthalein.

Place exactly N NaOH in a burette and add to the HCl until the appearance of the first permanent pink color against a white background.

Repeat titrations until at least two come within 0.05 cc. of each other.

Calculate normality of solution using the following formula:

$N_1 \times V_1 = N_2 \times V_2$.
N_1 = normality of HCl.
V_1 = amount of HCl used for the titration.
N_2 = normality of NaOH used.
V_2 = amount of NaOH used in the titration.
Example:
N(HCl) × 10 cc. = 1 N × 10.2 cc. NaOH.
N of HCl = 1.02 N.

To adjust to exact normality, use the above formula where:

N_1 = calculated normality of HCl.
V_1 = total cc. of remaining HCl.
N_2 = desired normality.
V_2 = final volume of HCl.
Example:
1.02 N × 970 cc. = 1 N × V_2.
V_2 = 989.4 cc.

It will be necessary to add 19.4 cc. (989.4 — 970) to make 1 N HCl.

6. If the solution is less than normal, more acid must be added which can be calculated as follows:
   a. Subtract the cc. for V_2 (value obtained in cc.) from the total remaining cc. of HCl (V_1).
   b. Calculate the amount of conc. HCl which is 11.8 N to be added as follows:
   cc. obtained in (a) × 1 N = (x) × 11.8 N.

C. ***Check on Normality.***

1. After the solution is adjusted, invert 100 times and let stand overnight.
2. Repeat titrations and if not exactly normal adjust as above and retitrate.

III. Sulfuric Acid.

A. ***Sulfuric Acid***—N/12.

1. Add 2.5 cc. of conc. H_2SO_4 (sp. gr. 1.84, 95.5%) to about 700 cc. of distilled water in a liter volumetric flask.

$$\text{The cc. of } H_2SO_4 \text{ used} = \frac{\dfrac{49 \text{ (eq. wt.)}}{12}}{1.84 \times 0.955}$$

2. Allow the solution to cool and then dilute to volume with distilled water at 20°C.
3. Invert 100 times and let stand overnight.
4. Standardize with exactly 0.1 N NaOH as described under 1 N HCl using phenolphthalein as an indicator.
5. It should take 16.67 cc. of 0.1 N NaOH to neutralize 20 cc. of N/12 H_2SO_4.
6. Adjust solution as described under N HCl and retitrate.

B. ***Sulfuric Acid***—2/3 N.

1. Add 20 cc. of conc. H_2SO_4 to about 700 cc. of distilled water in a liter volumetric flask.

$$\text{The cc. of } H_2SO_4 \text{ used} = \frac{2/3 \text{ of } 49}{1.89 \times 0.955}$$

2. Allow solution to cool and then dilute to volume with distilled water at 20°C.
3. Invert 100 times and let stand overnight.
4. Standardize with exactly N NaOH as described under N HCl using phenolphthalein as an indicator.
5. It should take 13.33 cc. of N NaOH to neutralize 20 cc. of 2/3 N H_2SO_4.
6. Adjust solution as described under N HCl and retitrate.

IV. Sodium Hydroxide.

A. ***Saturated Stock Solution.***

1. Weigh out 1100 gm. of reagent quality sodium hydroxide pellets on a rough balance and place in a 2 liter pyrex beaker.
2. With constant stirring, gradually add 1000

cc. of distilled water which has been freshly boiled and cooled.

3. Allow to cool, place in a pyrex bottle, and stopper with a paraffin-coated cork.
4. After the solution has stood for several days, the carbonate settles to the bottom leaving a clear supernatant liquid.
5. This solution contains approximately 74 gm. of sodium hydroxide for each 100 cc.

B. ***Normal Sodium Hydroxide.***

1. Place 55 cc. of the clear saturated NaOH solution in a liter volumetric flask. (A normal solution contains 40 gm. of NaOH; therefore, 40 divided by 0.74 will give the number of cc. of saturated solution needed.)
2. Dilute to volume at 20°C. with freshly boiled distilled water that has been cooled.
3. Invert 100 times and let stand overnight.
4. Standardize with exactly N HCl as described under N HCl using phenolphthalein as an indicator.
5. Adjust solution as described under N HCl and retitrate.

V. Sodium Thiosulfate Solution.

A. ***Sodium Thiosulfate Solution***—0.1 N.

1. Use distilled water that has been freshly boiled and cooled.
2. Place 25 gm. of c.p. sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in a liter volumetric flask and dissolve with about 700 cc. of water.
3. Dilute to volume at 20°C.
4. Shake thoroughly and let stand 2 weeks before standardizing.
5. Keep in a bottle with a siphon arrangement and carrying a soda lime tube to exclude CO_2.

B. ***Standardization.***

1. Standardize with a 0.1 N solution of acid potassium iodate [$KH(IO_3)_2$].

   $KIO_3 \cdot HIO_3 + 10\ KI + 11\ HCl \rightarrow 6\ I_2 + 11\ KCl + 6\ H_2O.$

   $I_2 + 2\ Na_2S_2O_8 \rightarrow 2\ NaI + Na_2S_4O_6.$

2. Weigh out exactly 3.2496 gm. of the purest c.p. grade of $KH(IO_3)_2$, place in a liter volumetric flask, and dilute to volume with distilled water at 20°C.
3. *Method of Titrating.*
   - a. Place 1 or 2 gm. of pure potassium iodide in a 500 cc. Erlenmeyer flask and dissolve in as little water as possible.
   - b. Add 5 cc. of 20% HCl and then add 25 cc. of 0.1 N $KH(IO_3)_2$.
   - c. Dilute at once with about 200 cc. of distilled water and titrate the liberated iodine immediately with the sodium thiosulfate solution dropping at a rapid rate and with continual agitation of the flask.
   - d. When the solution becomes pale yellow, add a few cc. of a 1% solution of soluble starch and titrate to loss of blue color.
   - e. Titrate until consistent checks are obtained.
   - f. Dilute the thiosulfate as follows:

$$\frac{25}{\text{cc. of thiosulfate used}} = \frac{X}{\text{volume of remaining thiosulfate}}$$

X = final volume of thiosulfate.

VI. Iodine Solution.

A. ***Iodine Solution***—0.1 N.

1. Dissolve 25 gm. of the best grade potassium iodide in about 250 cc. of distilled water.
2. Powder about 15 gm. of resublimed iodine to a very fine powder; weigh 12.7 gm. on a watch glass and dissolve in the KI solution. Do not add water to this until all of the iodine is dissolved.
3. Dilute to volume with distilled water and mix thoroughly.
4. Keep in a glass-stoppered brown bottle.

B. ***Standardization.***

1. Place 25 cc. of the iodine solution in a 300 cc. Erlenmeyer flask containing about 100 cc. of distilled water.
2. Add exactly 0.1 N sodium thiosulfate from a burette until a very faint yellow color remains.
3. Add 1 cc. of a 1% solution of soluble starch and add the thiosulfate solution drop by drop until the last trace of blue is removed.
4. Calculate the correction factor of the iodine solution.
5. This solution must be restandardized frequently.

Indicators

I. Alizarin Red.

Sodium alizarin monosulfonate	1.0 gm.
Distilled water	100.0 cc.

pH range 5.0—6.8.
Color change yellow to red.

II. Bromcresol Green.

Bromcresol green	40.0 mg.
Absolute ethyl alcohol	100.0 cc.

pH range 4.0—5.6.
Color change yellow to blue.

III. Bromthymol Blue.

Dibromthymolsulfonphthalein	40.0 mg.
Absolute ethyl alcohol	100.0 cc.

pH range 6.0—7.6.
Color change yellow to blue.

IV. Chlorphenol Red.

hlorphenol red	40.0 mg.
bsolute ethyl alcohol	100.0 cc.

H range 6.0—6.6.

olor change yellow to red.

V. Congo Red.

ongo red	0.5 gm.
istilled water	90.0 cc.
hyl alcohol (95%)	10.0 cc.

H range 3.0—5.0.

olor change blue to orange red.

VI. Dimethylaminoazobenzene (Töpfer's Reagent).

dimethylaminoazobenzene	0.5 gm.
hyl alcohol (95%)	100.0 cc.

H range 2.9—4.0.

olor change red to yellow.

VII. Methyl Orange.

ethyl orange	100.0 mg.
stilled water	100.0 cc.

H range 3.1—4.4.

olor change orange red to yellow.

VIII. Methyl Red.

ethyl red	1.0 gm.
hyl alcohol (95%)	300.0 cc.

lute to 500 cc. with distilled water.

H range 4.2—6.3.

olor change red to yellow.

IX. Neutral Red.

eutral red	0.5 gm.
hyl alcohol (95%)	300.0 cc.

lute to 500 cc. with distilled water.

H range 6.8—8.0.

olor change red to yellow.

X. Phenolphthalein.

enolphthalein	1.0 gm.
hyl alcohol (95%)	100.0 cc.

H range 8.3—10.0.

olor change colorless to red.

XI. Phenol Red.

enol red (phenolsulfonphthalein)	20.0 mg.
hyl alcohol (95%)	100.0 cc.

H range 6.6—8.2.

olor change yellow to red.

XII. Starch Solution.

luble starch	1.0 gm.
stilled water	100.0 cc.

Add enough of the cold distilled water to make hin paste and to this, while stirring, add boiling ater to make 100 cc.

XIII. Thymol Blue.

ymol blue (thymolsulfonphthalein)	40.0 mg.
solute ethyl alcohol	100.0 cc.

H range 1.2—2.8; color change red to yellow.

H range 8.2—9.8; color change yellow to blue.

Stains

I. Crystal Violet Solution.

Solution A.

Crystal violet	10 gm.
Ethyl alcohol (95%)	100 cc.

B. *Solution B.*

Ammonium oxalate, c.p.	10 gm.
Distilled water	1000 cc.

C. *Final Solution.*

Solution A	100 cc.
Solution B	800 cc.

Keeps well.

II. Carbol-Fuchsin Solution.

A. *Ziehl-Neelsen Stain for Tubercle Bacilli.*

Basic fuchsin (sat. alcoholic sol., see below)	10 cc.
Carbolic acid (5% aqueous sol.)	90 cc.

Make a saturated alcoholic solution of basic fuchsin as follows:

1. Add 30 cc. of 95% ethyl alcohol to 9 gm. of basic fuchsin.
2. Place the container in a water bath and heat to boiling for 2 minutes.
3. Allow to stand 1 minute and pour off supernatant fluid into a bottle.
4. Add a second 30 cc. of alcohol to the sediment, boil, and decant as before.
5. Add 34.5 cc. of alcohol to the sediment, boil. and decant as before.

B. *Dilute Carbol-Fuchsin Stain.*

Ziehl-Neelsen Stain	10 cc.
Distilled water	90 cc.

III. Gram's Iodine Solution.

Iodine crystals	1 gm.
Potassium iodide	2 gm.
Distilled water	300 cc.

IV. Loeffler's Alkaline Methylene Blue Solution.

Methylene blue chloride [sat. 95% alcoholic sol. (1.5%) filtered]	30 cc.
KOH (0.01% aqueous solution)	100 cc.

V. Lugol's Solution.

Iodine crystals	5 gm.
Potassium iodide	10 gm.
Distilled water	100 cc.

VI. Safranin Solution.

Safranin (2.5% in 95% alcohol)	10 cc.
Distilled water	90 cc.

VII. Sudan III.

Saturated solution (1%) of Sudan III in 70% ethyl alcohol.

TABLE 86. RANGE OF INDICATORS

Indicator	pH range	Color change
Thymol blue (acid range)	1.2–2.8	Red-yellow
Topfer's reagent	2.9–4.0	Red-yellow
Congo red	3.0–5.0	Blue-red
Methyl orange	3.1–4.4	Orange red-yellow
Bromcresol green	4.0–5.6	Yellow-blue
Methyl red	4.2–6.3	Red-yellow
Alizarin red	5.0–6.8	Yellow-red
Chlorphenol red	6.0–6.6	Yellow-red
Bromthymol blue	6.0–7.6	Yellow-blue
Phenol red	6.6–8.2	Yellow-red
Neutral red	6.8–8.0	Red-yellow
Thymol blue (alkaline range)	8.2–9.8	Yellow-blue
Phenolphthalein	8.3–10.0	Colorless-red

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